Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L.

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Departamento de Botánica
Tese de Doutoramento
Universidade de Santiago de Compostela
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Dra. **M. ISABEL FRAGA VILA**, PROFESORA TITULAR DE UNIVERSIDADE DO DEPARTAMENTO DE BOTÁNICA DA FACULTADE DE BIOLOXÍA DA UNIVERSIDADE DE SANTIAGO DE COMPOSTELA,

INFORMA:

Que a presente memoria titulada "**Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L."** presentada por **D. Celestino Quintela Sabarís** para optar ó Grao de Doutor en Bioloxía, foi realizada baixo a miña dirección no Departamento de Botánica da Universidade de Santiago de Compostela.

E considerando que representa traballo de Tese de Doutoramento, autorizo a súa presentación ante o Tribunal correspondente.

E para que así conste, asino a presente en Santiago de Compostela a 22 de Marzo de 2011.

Vº e Prace da Directora,
Asdo.: Dra. M. Isabel Fraga Vila

O Doutorando,
Asdo.: D. Celestino Quintela Sabarís

Aos meus pais e avós, responsáveis polo autor
A Berta e Martinho, que me lembram cada dia o que é importante na vida
A Bibi.

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RESUMO EN GALEGO DA TESE DE DOUTORAMENTO
Orixe evolutiva e ecofisioloxía das poboacións metalícolas de *Cistus ladanifer* L.

Introdución e antecedentes:

Os solos metalíferos (aqueles que conteñen cantidades elevadas de metais) son tóxicos para a maioría das plantas e outros organismos vivos. Dentro dos solos metalíferos, aqueles resultado da polución por actividades humanas, especialmente por actividades relacionadas coa produción de metais (entulleiras de minas, áreas de fundición...) constitúen unha ameaza para o medio ambiente e a saúde pública: normalmente teñen unha escasa (ou mesmo ausente) cobertura vexetal e, polo tanto, os metais poden sofrer unha filtración cara as augas subterráneas, ou o propio solo poluído pode dispersarse polo vento e afectar terras agrícolas produtivas ou áreas protexidas (Ruttens *et al.* 2006, Tordoff *et al.* 2000).

Un resultado desta preocupación pública foi a promulgación pola Comisión Europea da Estratexia Europea Temática para a protección dos Solos (COM (2006) 231) que inclúe unha proposta de directiva marco sobre os solos. Un dos principais obxectivos desta estratexia é a recuperación de solos degradados, sendo a contaminación por metais pesados unha das causas principais da degradación do solo. Estímase que no seo da UE, existen preto de 0,5 millóns de lugares contaminados que precisan ser recuperados (SEC (2006) 1165).

As tecnoloxías clásicas para a recuperación de solos adoitan implicar procesos que son caros e moitas veces destrutivos (solidificación e estabilización, lavado do solo, electrocinética, redución/oxidación química, incineración, vitrificación, escavación e eliminación en vertedoiro, etc) (Wenzel *et al.* 2004).

Porén, o uso de plantas para a recuperación de lugares contaminados con metais (Fitorremediación) está gañando unha importancia considerábel, xa que a fitorremediación é considerada "menos invasiva e máis eficaz na restauración da estrutura do solo e das funcións en comparación cos métodos de enxeñería civil" (Kidd *et al.* 2009).

Centrándonos no caso dos metais pesados, a base de todas estas fitotecnoloxías é a existencia, identificación e descrición de especies vexetais (ou poboacións dentro de determinadas especies), que sexan tolerantes a metais pesados. É dicir, plantas que teñan a capacidade de colonizar, sobrevivir e reproducirse nun solo con elevadas concentracións de metais pesados, tóxico para a maioría das outras plantas (Antonovics *et al.* 1971, Macnair 1993).

Baker (1981) estableceu que, de acordo co seu patrón de resposta aos

metais, as especies tolerantes pódense clasificar en tres grupos principais: acumuladoras, indicadoras e exclusoras.

Así, unha exclusora presenta baixos niveis de metais na súa parte aérea para ampla gama de concentracións externas de metais, até un valor crítico no solo a partir do cal o mecanismo de exclusión quebra, o que resulta nunha falta de restricción do transporte do metal cara a parte aérea. En contraste, as especies acumuladoras concentran os metais na parte aérea, haxa unha concentración alta ou baixa de metal no solo, mentres que as plantas indicadoras mostran unha relación proporcional entre os niveis de metais no solo e a súa acumulación na parte aérea. Unha categoría adicional comprende as especies hiperacumuladoras: plantas que se comportan de forma semellante ás acumuladoras, mais cun factor de acumulación moito máis elevado.

Desde o punto de vista da súa distribución respecto ás áreas con metais pesados, podemos clasificar as especies vexetais en tres tipos (Pollard *et al.* 2002): non-metalófitos estrictos (especies restrinxidas a solos con baixo contido de metais, que é o caso da gran maioría das plantas), metalófitos estrictos (plantas que medran exclusivamente en solos metalíferos, como entulleiras de minas ou áreas serpentínicas) e metalófitos facultativos, tamén chamados pseudometalófitos (especies con poboacións que medran en solos metalíferos e non metalíferos).

Dentro dos pseudometalófitos podemos distinguir dous subtipos: por unha banda, as especies con tolerancia constitutiva (todos os xenotipos son tolerantes a metais, mesmo naquelas poboacións que medran en solos con baixo contido de metais) e do outro lado, as especies con variedades ou ecotipos tolerantes aos metais que evolucionaron durante a colonización de áreas metalíferas a partir de poboacións onde os xenotipos tolerantes estaban presentes en baixa frecuencia (Pollard *et al.* 2002).

Para completar este esquema, relativamente simple, tamén se ten citado o caso de especies vexetais que, con independencia da súa tolerancia a metais, grazas a posuír preadaptacións a algunha das condicións edáficas estrictas dos solos metalíferos (falta de nutrientes ou desequilibrio nos mesmos, estrés hídrico, alta insolación, etc) poden colonizar estas áreas máis facilmente (Brady *et al.* 2005; e referencias citadas) de xeito que, tal como Wu (1990) describiu, é posíbel a colonización de solos metalíferos sen a evolución da tolerancia.

De cara á posta en práctica de procesos de fitorremediación, os pseudometalófitos posúen un elevado interese, xa que alén da súa tolerancia aos metais, posúen outras características importantes, como son a súa alta capacidade de adaptación a unha ampla gama de condicións adversas do solo, xunto coa súa elevada produción de biomasa e a súa competitividade en solos con moderada toxicidade por metais (Poschenrieder *et al.* 2001).

Alén disto, e dadas as diferenzas en tolerancia que podemos topar entre poboacións dentro dunha especie de pseudometalófito, estes organismos permíten-

nos investigar a xenética e fisioloxía das diferenzas entre as plantas tolerantes e non tolerantes dunha forma que xeralmente non é posíbel cos edafo-endemismos (Macnair 1993). Ademais, as poboacións metalícolas (M) e non-metalícolas (NM) veciñas dun determinado pseudometalófito son modelos relevantes para o estudo da adaptación local en plantas (Linhart e Grant 1996).

A esteva ("gum-rockrose" en inglés ou"jara pringosa"en castellano-español) (*Cistus ladanifer* L., Fam Cistaceae) é un arbusto leñoso presente na Rexión Mediterránea Occidental (desde o sur de Francia até o Norte de Marrocos e Alxeria) (Demoly e Montserrat 1993).

Un exemplar adulto de *C. ladanifer* pode acadar os 2 m de altura, con sistemas radicais e aéreos densos (Martín Bolaños e Guinea López 1949). Posúe follas lanceoladas verdes cunha cara glabra e o envés cuberto cun tomento branco. As follas presentan unha disposición decusada e están soldadas entre sí pola base. A morfoloxía foliar emprégase como base para a identificación das súas tres subespecies: subspp. *africanus*, *ladanifer* e *sulcatus* (Demoly e Montserrat 1993). A subsp. *africanus* é a única con follas pecioladas, namentres nas subspp. *ladanifer* e *sulcatus* son sésiles. Ademais, na subsp. *sulcatus* (orixinalmente descrita como *Cistus palhinhae*) a superficie da folla está dividida por nervios ben marcados.

A subsp. *ladanifer* distribúese principalmente na Península Ibérica, norte de África e Francia (onde é considerada unha especie introducida), subsp. *sulcatus* é endémica para o sudoeste de Portugal (rexión do Algarve) e subsp. *africanus* está presente no sur de España (Cádiz, Málaga), pero é máis común no norte de África (Demoly e Montserrat 1993). Guzmán e Vargas (2009) dataron a orixe de *C. ladanifer* e mais a diverxencia das súas diferentes subespecies durante o Pleistoceno Superior.

As súas flores son grandes (55-70 mm de diámetro) e solitarias, con 3 sépalos e 5 pétalos brancos (var. *albiflorus*) que tamén pode ter unha mancha de cor vermella a acastañada na base (var. *maculatus*). Cada flor ten un estigma sésil e un gran número de estames que poden producir máis de 700.000 grans de pole por flor (Talavera *et al.* 1993).

A polinización é entomófila, e posúe un mecanismo gametofítico de autoincompatibilidade, polo que a esteva é unha especie alógama obrigada (Talavera *et al.* 1993). Este feito, xunto cunhas distancias de dispersión de pole curtas (poucos metros) resulta nunha redución da eficiencia reprodutiva en exemplares isolados (Metcalfe e Kunin 2006). O froito é unha cápsula loculicida, con 5-7 a 12 lóculos. Unha única cápsula pode conter até 1.000 sementes, polo que se estima que un adulto de *C. ladanifer* pode producir máis de 158.000 sementes, que se liberan durante un período de 8 a 10 meses desde mediados do verán (Bastida e Talavera 2002). A dispersión das sementes é principalmente barócora, e máis do 80% da caída de sementes ocorre baixo a copa das plantas nai (Bastida e Talavera 2002), aínda que se ten estimado que diferentes

especies de formigas granívoras (xénero *Goniomma*) e o cervo (*Cervus elaphus*), que se alimentan de sementes e froitos respectivamente, poden desempeñar un papel importante na dispersión de sementes a longa distancia (Bastida e Talavera 2002, Malo e Suárez 1998).

As poboacións de *C. ladanifer* constitúen as etapas iniciais da sucesión ecolóxica nos ecosistemas mediterráneos. Estas poboacións están adaptadas ás alteracións destes ecosistemas, especialmente ao lume: as súas sementes manteñen unha elevada viabilidade durante varios anos (sementes de 3 anos de idade amosaron unha taxa de xerminación por riba do 80%, observación persoal, C. Quintela-Sabarís) e posúen un mecanismo de dormición física que pode ser interrompida polo lume, as altas temperaturas (Pérez-García 1997), o fume ou as sales nitroxenadas (Pérez Fernández e Rodríguez Echeverría 2003).

Durante o primeiro ano post-incendio, ocorre unha xermolación masiva de plántulas (Ferrandis *et al.* 1999), que permite a rápida rexeneración das poboacións orixinais (dous anos despois dunha queima experimental, *C. ladanifer* cobre o 40% da área orixinal, Calvo *et al.* 2005).

Ademais, esta especie está ben adaptada ao estrés hídrico e ao exceso de insolación. Posúe un sistema radical denso e superficial que favorece a captación de auga nas limitadas épocas de chuvia (Martín Bolaños e Guinea López 1949). Alén disto, é semi-decídua, mantendo durante o período de seca estival follas de pequeno tamaño potencialmente activas (Núñez- Olivera *et al.* 1996). As follas tamén contan cunha fotoprotección grazas á exudación dunha resina perfumada e pegañenta (o ládano), que aumenta durante o verán (Chaves *et al.* 1993).

Dado o seu contido en flavonoides e outros compostos fenólicos do metabolismo secundario, a exudación de ládano ten efectos positivos adicionais na esteva: o ládano é un composto con propiedades alelopáticas que inhibe a xermolación doutras plantas (Herranz *et al.* 2006) e proporciona unha defensa contra a inxestión por parte de herbívoros ao inhibir neles a relaxación dos músculos esqueléticos da boca (Sosa *et al.* 2004).

En relación aos solos, *C. ladanifer* é o principal compoñente de matogueiras desenvolvidas en solos ácidos oligotróficos da metade occidental da Península Ibérica (Rivas-Martínez 1979), se ben modelos de distribución apoian a súa tolerancia a solos calcáreos (Gastón *et al.* 2009) e a subsp. *sulcatus* está restrinxida a solos derivados de caliza no litoral sudoeste de Portugal.

A súa capacidade de medrar en áreas pobres en nutrientes pode ser favorecida polo establecemento de relacións simbióticas con microorganismos nas súas raíces. Varias cepas de bacterias relacionadas coa solubilización de fosfato ou a produción de sideróforos (un deles identificado como rizobacteria promotora de crecemento vexetal- PGPR), foron illadas a partir de raíces de *C. ladanifer* (Ramos Solano *et al.* 2006). Ademais, na literatura científica existe referencia da identificación de máis de 30 especies de fungos que forman

relacións simbióticas (ectomicorrizas) con *C. ladanifer* (Comandini *et al.* 2006).

Pero o máis importante entre todas estas características interesantes é o feito de que ***C. ladanifer* é un pseudometalófito**. As subespecies *ladanifer* e *africanus* teñen establecido con éxito poboacións en áreas serpentínicas ou en entulleiras de mina onde, nalgúns casos, son a especie dominante. A presenza de *C. ladanifer* sobre solos metalíferos, e a acumulación de metais en follas desta planta está reflectida nunha ampla produción científica. Nalgúns destes traballos (Alvarenga *et al.* 2004, Pratas *et al.* 2005, Murciego Murciego *et al.* 2006), a esteva foi descrita como especie indicadora ou mesmo acumuladora de As, Sb e Zn, e Mn, Sb e W, respectivamente.

Estas referencias proporcionan información útil sobre a esteva e os metais, aínda que, ao ser estudos a nivel local ou rexional, mesmo empregando diferentes metodoloxías de mostraxe e cuantificación de metais, é difícil facer comparacións entre eles.

No relativo á tolerancia de *C. ladanifer* aos metais, só temos constancia de tres traballos científicos previos, cada un deles usando diferentes enfoques.

• Alados *et al.* (1999), usando unha análise de estabilidade no desenvolvemento, demostraron a adaptación de *C. ladanifer* aos solos serpentínicos de Málaga (S de España). Estes autores presentan a hipótese de que o baixo requerimento de Ca^{2+} por parte desta especie pode ser unha vantaxe na colonización de áreas serpentínicas. Nesta mesma liña, Ater *et al.* (2000) cuantificaron unha ratio Mg/Ca elevada en follas de *C. ladanifer* crecendo en zonas serpetínicas do norte de Marrocos.

• Kidd *et al.* (2004) someteron a plántulas de 5 de poboacións de solos metalíferos e non metalíferos do NE de Portugal a experimentos de tolerancia ao Cd, Co, Cr, Mn, Cu, Ni, Pb e Zn en cultivo hidropónico. Observaron patróns de tolerancia e acumulación de metais específicos para cada poboación, e estimaron que mesmo poboacións non-metalícolas posuían unha certa tolerancia aos metais pesados.

• Santos *et al.* (2009) procuraron actividade diferencial de enzimas antioxidantes en plantas de *C. ladanifer* dunha zona de minas abandonadas en SE Portugal, pero non atoparon ningunha variación relacionada especificamente cos metais.

Nunha Tese de Doutoramento de elevado interese, Díez-Lázaro (2008) tratou a optimización do uso de *Cistus ladanifer* para procesos de fitorremediación. Encontrou que a adición de fertilizantes e a acidificación do solo melloraron o crecemento e a extracción de Mn e Zn por plantas desta especie procedentes do Nordeste de Portugal. Ademais, estableceu que a esteva podería ser utilizado para a fitoextracción de Zn en solos con contido baixo-medio deste metal.

Finalmente, o efecto beneficioso desta planta é subliñado por Simões *et al.* (2009), quen estimaron que pode producir máis de 4.600 kg de follada·ha^{-1}·ano^{-1}, o que mellora a calidade do solo e pode promover a rexeneración da vexetación ao facilitar a colonización dunha área por es-

pecies con requerimentos máis esixentes.

En resumo, *C. ladanifer* posúe unha serie de características (adaptación á seca, baixo requerimento de nutrintes, tolerancia a metais en certas poboacións) que a fan especialmente útil para a recuperación de áreas degradadas na rexión mediterránea. Ademais, é unha especie nativa desta rexión rica en biodiversidade e, polo tanto, o seu uso non implica efectos prexudiciais sobre os ecosistemas circundantes producido polo emprego de especies exóticas e invasoras (Méndez e Maier 2008, e referencias citadas nese traballo). Alén disto, *C. ladanifer* constitúe unha especie modelo interesante para o estudo do proceso de colonización de áreas metálicos por plantas.

OBXECTIVOS E TAREFAS DESENVOLVIDAS:

Dentro deste marco, e co obxectivo de mellorar o coñecemento sobre *Cistus ladanifer* e as súas relacións con metais, desenvolveuse unha campaña de recollida de mostras de planta (follas, sementes) e de mostras de solo en diferentes localidades do rango desta especie (Rexión Mediterránea Occidental, principalmente Península Ibérica e Norte de Marrocos). Buscouse cubrir diferentes tipos de material xeolóxico, dando especial atención ás áreas metalíferas (solos serpentínicos de Trás-Os-Montes, Málaga e Rif; áreas mineiras do SW da Península Ibérica), resultando nun total de 33 poboacións. O material recolectado empregouse nunha serie de investigacións, desenvolvidas a fin de tratar os seguintes temas:

• Un primeiro paso para inferir os efectos dos metais sobre a xenética da especie é entender a interacción de procesos que determinan a súa filoxeografía ou "paisaxe xenética". Dentro dos diferentes tipos de marcadores moleculares, aqueles baseados no ADN do cloroplasto (cpADN, ben sexan microsatélites do cloroplasto-cpSSRs ou PCR-RFLPs) teñen sido amplamente empregados en estudos de filoxeografía de plantas (Petit *et al.* 2003, Magri *et al.* 2007) ao posuír unha serie de características útiles:

- Nas anxiospermas, o cpADN polo xeral hérdase por vía materna, polo que a súa dispersión realízase soamente através das sementes, resultando nunha mellor estruturación xeográfica das súas variedades.

- Ao herdarse por vía materna, os patróns xeográficos do cpADN non se ven influídos polo fluxo de pole entre poboacións.

- Ademais, o cpADN é haploide, polo que o seu tamaño efectivo de poboación é menor que o dos xenes nucleares (diploides). Destes xeito, a diferenciación por deriva xenética pode ser máis forte (Comes e Kadereit 1998) e fenómenos como colos de botella xenéticos poden ser detectados de xeito máis doado (Echt *et al.* 1998) co cpADN que cos xenes nucleares. Por exemplo, os marcadores cpSSR detectaron que as poboacións de *Silene paradoxa* medrando en entulleiras de minas de cobre sufriran unha redución na súa diversidade xenética (Mengoni *et al.* 2001), en canto un estudo

previo con RAPD sobre esas mesmas poboacións fallara na detección desa baixa diversidade (Mengoni *et al.* 2000).

- Finalmente, os microsatélites do cloroplasto son considerados marcadores neutros, é dicir, todos os seus alelos (variantes) teñen efectos iguais sobre o individuo que os transporta. Así, os cpSSRs proporcionar unha información independente da selección que nos permite separar os efectos da filoxeografía dos efectos da contaminación por metais.

É por isto que desenvolvemos un estudo a grande escala da filoxeografía de *C. ladanifer* empregando cpSSRs (capítulo 2).

• Posteriormente cuantificamos, empregando fluorescencia de raios X (XRF), ICP-masas e espectrometría de absorción atómica (AAS), os contidos totais e extraíbeis de metais nos solos de 33 poboacións procedentes de case todo o rango de *C. ladanifer*. En base a esta información, clasificamos esas poboacións como metalícolas (que medran en solos con contidos elevados de metais, abreviado M) e non-metalícolas (que medran en solos "normais", abreviado NM). O tipo de poboación integrouse coa información filoxeográfica dos cpSSRs (ver capítulo 3) para respostar ás seguintes preguntas: As poboacións M teñen unha orixe mono ou polifilética? A colonización de áreas metalíferas provoca perda de diversidade xenética?

• Un seguinte paso foi cuantificar a acumulación de metais pesados en follas de esteva recollidas no campo e procedentes das 33 poboacións consideradas no capítulo anterior; para o que empregamos XRF, ICP-OES e ICP-masas. Dado que a nivel xenético unha especie non é unha mestura homoxénea de alelos, senón que adoita estar dividida en subgrupos, no caso de que as poboacións M teñan unha orixe evolutiva múltiple, é posíbel que existan diferencias nas estratexias de resposta aos metais (Gonnelli *et al.* 2001, Nyberg Berglund *et al.* 2003). Consideramos este aspecto ao computar índices de bioacumulación de metais en cada poboación e avaliar o efecto do tipo de solo (metalífero ou non metalífero) e a información filoxeográfica sobre os patróns de acumulación (contidos totais e relativos de metais nas follas) (ver capítulo 4).

• Alén da resposta en acumulación en campo, desenvolvemos experimentos de invernadoiro en condicións de cultivo hidropónico para avaliar a tolerancia de *C. ladanifer* aos metais Co, Ni e Zn (ver capítulo 5). Estimamos a tolerancia en base a medidas de crecemento (lonxitude da raíz mais longa, lonxitude do caule e incremento de número de follas), biomasa (peso seco) e eficiencia fotosintética ("yield"). Transformamos estas variábeis en medidas relativas computadas como unha porcentaxe dos valores obtidos para as plantas control, segundo a proposta orixinal de Wilkings (1978) para o crecemento da raíz. Así, eliminamos a maior parte da variación nas respostas non relacionadas cos tratamentos con metais.

• Un último paso, interesante para o desen-

volvemento de futuras investigacións, é a identificación de marcadores potencialmente ligados á tolerancia a solos metalíferos. Isto é especialmente necesario en plantas non-modelo, como *C. ladanifer*. Abordamos este tema aproveitando as posibilidades de desenvolvemento de escáneres do xenoma ("genome scans") dos AFLP (siglas do inglés: Amplified Fragment Length Polymorphism) (Vos *et al.* 1995). Estes marcardores moleculares fornecen información do xenoma nuclear e permiten obter centos de marcadores potencialmente non ligados dunha especie determinada sen ter un coñecemento previo das secuencias do seu ADN. Os AFLPs teñen sido empregados en organismos non-modelo (Bonin *et al.* 2007, e referencias dese traballo). En concreto, utilizáronse para detectar loci potencialmente implicados na adaptación a diversas condicións ambientais en plantas (Namroud *et al.* 2008, Parisod e Christin 2008, Poncet *et al.* 2010), e mesmo adaptación a metais pesados en poboacións do pseudometalófito *Arabidopsis halleri* (Meyer *et al.* 2009). Nesta tese de doutoramento, optimizamos un protocolo de análise de *C. ladanifer* con AFLP, obtendo un elevado número de marcadores. Posteriormente, comparamos estes marcadores coa información dispoñíbel sobre os solos (pH, ratios Ca:Mg e contidos de metais pesados), mediante a aplicación de Ecuacións de Estimación Xeralizadas (GEE, en inglés) (ver capítulo 6). As GEE, que son unha extensión dos modelos lineares xeralizados (Carl e Kuhn 2007), permiten analizar os modelos de distribución de alelos e estimar as variábeis ambientais que teñen unha maior influencia sobre eses modelos de distribución. Ademais, as GEE permiten introducir e corrixir a posíbel autocorrelación entre as mostras procedentes dunha mesma poboación ou dunha mesma liñaxe cloroplastidial. Finalmente, realizamos unha comparación das inferencias da estrutura xenética das poboacións de *Cistus ladanifer* obtidas con AFLPs (marcadores nucleares, diploides, de herdanza biparental e que poden sufrir recombinación) e cpSSRs (marcadores cloroplastidiais, haploides, de herdanza materna, que non sofren recombinación e entón hérdanse como bloques-haplotipos).

SÍNTESE E CONCLUSIÓNS:

A seguir presentamos unha síntese final e conclusións elaboradas en base aos resultados obtidos a partir dos experimentos expostos nos parágrafos anteriores:

Grazas á análise a grande escala de *Cistus ladanifer* empregando microsatélites de cloroplasto (cpSSRs), inferimos que a diversidade xenética desta especie con grande rango de distribución está na realidade estructurada en dúas (ou tres, dependendo da metodoloxía estatística empregada) liñaxes ou clusters principais.

Atendendo aos datos de pole presentes na bibliografía, estes conxuntos son o resultado da recolonización posglacial da Rexión Mediterránea Occidental a partir de refuxios illados e situados no norte de Marrocos e no sudoeste e sueste da Península Ibérica. Así mesmo, temos que

concluír que as poboacións metalícolas desta especie son o resultado de múltiples e independentes procesos de colonización. Curiosamente, a colonización de áreas metalíferas non deixou pegadas xenéticas (na forma de colos de botella xenéticos ou efecto fundador) relacionadas co tipo de solo. Este feito constitúe un primeiro apoio á tolerancia a metais pesados como unha característica 'constitutiva' (presente en toda a especie) da esteva.

Hai un gran número de estudos locais e rexionais sobre a acumulación de metais pesados por *C. ladanifer*. A aplicación dos coñecementos filoxeográficos permitiunos separar, nun estudo a nivel de toda a especie, os efectos da liñaxe cloroplastidial e do solo. Ao compararmos as poboacións metalícolas (M) e non-metalícolas (NM), non observamos diferenzas entre elas nos contidos foliares de diferentes metais, agás Ni. Unha explicación plausible é a aparición, nas poboacións M, de mecanismos de restricción da acumulación de metais. A pesar da afirmación anterior, atopamos diferentes patróns de acumulación de metais pesados entre as poboacións M de liñaxes cloroplastidiais diferentes. Este fenómeno, xa observado noutras especies de pseudometalófitos, reflicte e serve de apoio a unha historia de evolución independente das poboacións M, que evolucionaron en paralelo dentro de liñaxes que se mantiveron illadas desde o Último Máximo Glacial (que rematou aprox. 20.000 anos antes do presente).

Con todo, e a pesar da esteva rexeitar claramente a acumulación dos metais Co, Cr e Pb, temos notado diferenzas significativas na resposta a outros metais entre as poboacións a nivel individual. Disto podemos derivar que calquera procedemento de fitoestabilización que implique o uso desta especie debe ser precedido por unha investigación que permita a caracterización dos seus ecotipos locais respecto dos metais pesados, a fin de evitar unha transferencia de metais (mediada pola esteva) do solo cara a rede trófica do ecosistema.

Os experimentos de tolerancia a Co, Ni e Zn, en condicións de cultivo hidropónico, revelaron que cada metal afecta de xeito diferente á esteva. Ademais, os efectos de cada metal son congruentes cos patróns de acumulación/exclusión que observamos para cada metal a partir de mostras tomadas no campo. Así, en futuras análises de tolerancia a metais en plantas sería útil coñecer a estratexia de resposta aos metais pesados dunha determinada especie, a fin de determinar o mellor parámetro (estimador de tolerancia) a ser medido.

Nas condicións do noso experimento non foron observadas diferenzas entre as poboacións M e NM para a meirande parte das variábeis resposta (crecemento, biomasa, fluorescencia clorofílica). Este feito pode ser interpretado como un segundo apoio para a tolerancia a metais como un carácter constitutivo de *C. ladanifer*. Unha outra vez, as diferentes liñaxes cloroplastidiais implican diferentes patróns ou mecanismos de resposta aos metais. Con todo, e dado o efecto dos tratamentos con metais sobre as variábeis resposta, suxerimos que pre-adaptacións á

escaseza de nutrientes ou ao estrés hídrico, en lugar dunha verdadeira tolerancia aos metais, poden ter facilitado a colonización de solos metalíferos por *C. ladanifer*.

Os marcadores AFLP, aínda posuíndo diferentes propiedades que os cpSSRs (xenoma diploide de herdanza biparental fronte a xenoma haploide de herdanza materna) forneceron as mesmas inferencias sobre a filoxeografia da especie. Así mesmo, os AFLPs non demostraron unha influencia do tipo de solo sobre a diversidade xenética e a diferenciación entre poboacións desta especie.

O procedemento de GEE resultou ser unha ferramenta estatística útil que nos permitiu analizar en conxunto os datos moleculares e a información sobre o solo. De acordo coas evidencias proporcionadas por diferentes autores sobre as esixencias nutricionais de *C. ladanifer*, a relación Ca: Mg (un dos factores de estrés máis importantes nas áreas serpentínicas, Brady *et al.* 2005) non tivo ningún efecto sobre a distribución de marcadores AFLP. Porén, verificamos que, entre todas as variábeis consideradas, o contido de Mn en solos ten o efecto máis forte na distribución de alelos. De feito, temos detectado unha banda cun posíbel papel na tolerancia ao alto contido de Mn no solo, aínda que precisamos futuras investigacións que permitan estimar o valor adaptativo da mesma.

Como apartado adicional, suxírense algunhas posíbeis liñas de futuras investigacións, que se verían favorecidas polos coñecementos derivados desta tese de doutoramento: i) estudo da implicación de simbioses planta-microorganimos (fungos ectomicorrízicos, rizobacterias) na tolerancia; ii) estudos sobre a colonización de áreas metalíferas a nivel da xenética da paisaxe e iii) integración dos datos filoxeográficos en estudos de quimioecoloxía (variación nos exudados foliares da esteva).

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Chapter 1

Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L.: Introduction and objectives.

Previous page: Close-up of a flower of *Cistus ladanifer* subsp. *ladanifer* var. *maculatus* (with a spot on each petal), growing on ultramafic soils in Samil (Trás-Os-Montes, NE Portugal). Flowers of *C. ladanifer* produce high amounts of pollen and nectar that attract a diverse array of insects including beetles, flies and bees. (Photo: PS Kidd)

CHAPTER 1

Introduction and Objectives

Soils with high concentrations of metals (metalliferous soils) are toxic to most plants and other living organisms (Boxes 1.1 and 1.2). Moreover, soils polluted by human activities, particularly those activities related to the production of metals (mine tailings, smelting areas ...) constitute a threat to the environment and public health: they usually have sparse (or even absent) plant cover and thus metals can filter into groundwater or the polluted soil can be dispersed by wind and then affect productive agricultural land or natural reserves (Ruttens *et al.* 2006, Tordoff *et al.* 2000).

A result of this public concern is the promulgation of the European Thematic Strategy for Soil Protection (COM (2006) 231) by the European Commission, which includes a proposal for a Framework Directive on Soils. One of the main objectives of this Strategy is the restoration of degraded soils, with pollution due to heavy metals being one of the causes of soil degradation. It is estimated that within the EU there are around 0.5 million contaminated sites which need to be remediated (SEC (2006) 1165). Classic technologies for the remediation of soils involve processes that are expensive and often destructive (solidification and stabilization, soil flushing, electrokinetics, chemical reduction/oxidation, soil washing, incineration, vitrification, excavation/retrieval, landfill and disposal, etc) (Wenzel *et al.* 2004).

In contrast, the use of plants for the reclaiming of metal polluted sites (**phytoremediation**) is gaining considerable importance, since phytoremediation is considered to be "*less invasive, more cost-effective and restorative of soil structure and functions compared to civil-engineering methods*" (Kidd *et al.* 2009).

The term phytoremediation covers a number of phytotechnologies with different characteristics (Prasad 2004, Kidd *et al.* 2009; and references therein):

- **Phytostabilization**: in situ inactivation of metals using a combination of plants and soil amendments.
- **Phytoextraction**: use of plants to absorb metals from the soil into plant roots and in some cases, translocation to above-ground plant parts.
- **Phytovolatilization**: absorption and transformation of metals into non-toxic volatile forms by plants (applied specifically to Hg and Se), and
- **Rhizofiltration**: use of plant roots to absorb, concentrate and/or precipitate heavy metals from aqueous solutions.

Considering heavy metals, all these phytotechnologies are based on the occurrence of plant species (or populations within certain species) which are tolerant

BOX 1.1 What are heavy metals? Why are they toxic? Micronutrients?

'Heavy metals' is an artificial category in which metals that have a high affinity for organic molecules are grouped. This affinity has determined that in the evolution of life some of them have acquired an essential role in animal and plant metabolism (see table A for key elements in plants) but always in low concentrations. This subgroup of heavy metals are thus considered 'micronutrients' (Epstein and Bloom 2005).

However, when a heavy metal, still being an essential element, exceeds a certain 'threshold of safety' inside the organism (see Fig. A, lower), it causes toxic effects that may result in reduction of growth, decrease of reproductive performance ('fitness') or even the death of the organism. In the case of essential heavy metals, the organism may suffer negative effects if minimal concentrations are not reached (Fig. A, upper).

The toxicity mechanisms involve one or more of the following (Hall 2002): i) Displacement of essential metal ions from biomolecules, ii) Blocking of essential functional groups of biomolecules and modification of its active conformation, iii) Disruption of the integrity of biomolecules, iv) Modification of other biologically active agent, v) Stimulation of the formation of free radicals and reactive oxygen species (ROS).

Table A: heavy metals as micronutrients

Function	Elements
Photosynthesis	Cu, Fe, Mn
Detoxification of ROS	Cu, Fe, Mn, Zn
Growth regulation	Fe, Mn
DNA transcription	Zn
Nitrogen metabolism	Co, Ni, Mo

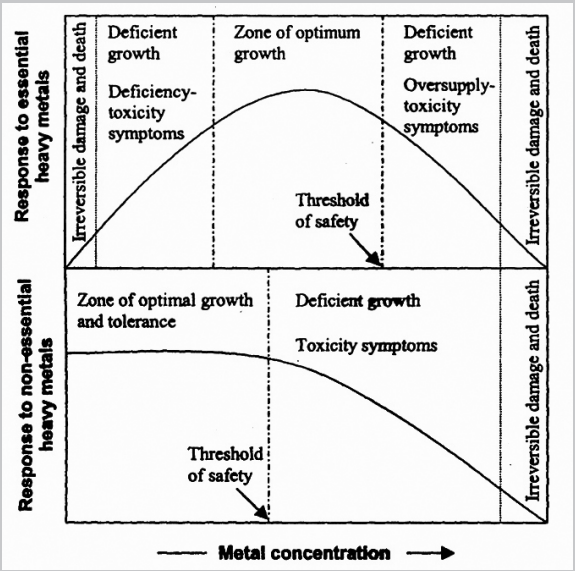

Fig. A: growth responses of plants to increasing concentrations of metals. From Shaw *et al.* (2004).

Although there are different definitions for heavy metals (Passow *et al.* 1961, Tiller 1989, Borovik 1990, Alloway 1995), we will follow Kidd *et al.* (2009) and will use interchangeably the terms 'heavy metal' or 'trace metal' throughout this document to refer to "*elements that occur in natural and perturbed environments in small amounts, and that, when present in sufficient bioavailable concentrations, are toxic to living organisms*".

BOX 1.2 Heavy metals in soils: sources, mobility and bioavailability.

The contents of heavy metals in undisturbed soils is mainly determined by the kind of parent material or bedrock and the processes of soil formation from it (Kidd *et al.* 2009, Greger 2004), together with processes of transport and deposition (atmospheric transport, …). Thus, although the mean background levels (amounts of heavy metals in soil not disturbed by human activity) are low, there are places where they may reach high concentrations (Table B).

As well as natural factors, human activities during recent millenia, and especially in the last century, have released an increasing amount of heavy metals into terrestrial and marine ecosystems. For some of these elements, the human effect has widely exceeded the natural sources. (Table B).

Element	Lithosphere	Serpentine	Calamine	Human/natural ratio of emissions
Cd	0.5		8	18.97
Co		180		
Cr		4384		
Cu	24	150	11	13.63
Hg	0.243			0.44
Ni	75	1574		3.46
Pb	31.5	21	3996	345.83
Zn	70	88	3403	23.46

Table B: Mean trace metal contents ($mg.kg^{-1}$) in Lithosphere and in serpentine and calamine soils. Ratio of human/natural emissions of metals to the Atmosphere. Modified from Friedland (1990).

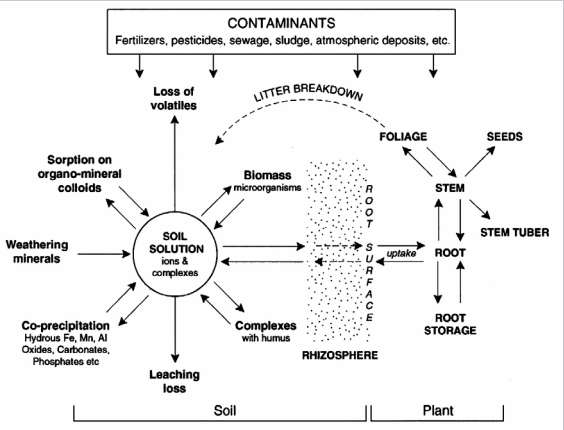

Fig. B: Plant-soil system. The most important factors related to availability and movement of metals are indicated. From Alloway (1995).

Once in soils, the mobility (retention/leachability) and bio-availability of metals is controlled by different soil properties (mainly pH, cationic exchange capacity, clay and organic matter content,) (Alloway 1995, Figure B). For most trace metals, which in soils are in the form of cations (positive charged ions), the lower clay and/or organic matter content and pH, the higher bio-availability.
In the fraction of soil surrounding plant roots (called rhizosphere) biological processes are predominant, and the collaboration of plants with different microorganisms (fungi and bacteria) may modify the metal bio-availability (Kidd *et al.* 2009).

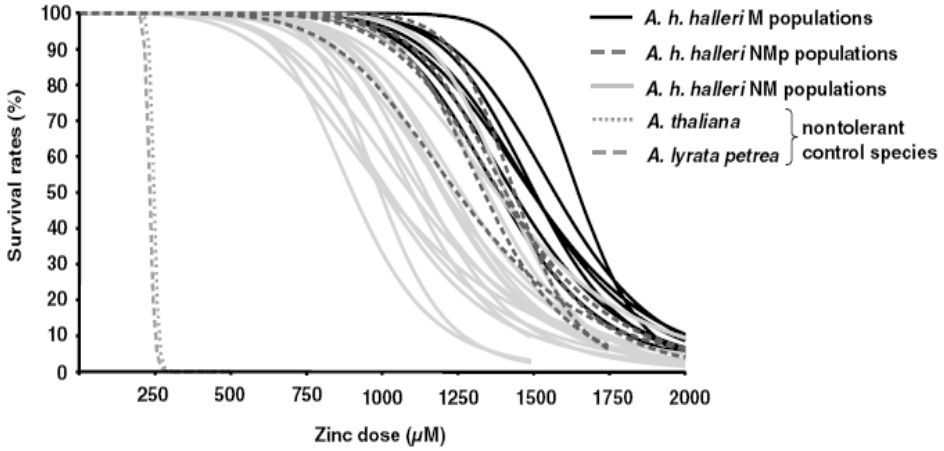

Figure 1.1: Response (measured as survival rates) of different populations of *Arabidopsis halleri*, *A. thaliana* and *A. lyrata* to increasing Zn doses. *A. halleri* populations are tolerant to Zn, whereas the other two *Arabidopsis* species are not. From Pauwels *et al.* (2006).

to heavy metals. That is, they have the capacity to colonize, survive and reproduce in a metal-polluted soil, toxic for most other plants (Antonovics *et al.* 1971, Macnair 1993).

Therefore, the recent interest in developing new lines for phytoremediation has added to the existing body of research on the mechanisms underlying heavy metal tolerance. It is desirable to identify tolerant plant species which also possess other interesting agronomic traits that make phytoremediation feasible (high biomass production, dense root and shoot systems, and even, in arid regions, adaptation to water stress; Frérot *et al.* 2006) or to dissect the genes responsive to tolerance and then transfer them (through biotechnology) into non-tolerant species with those interesting agronomic traits (Pauwels *et al.* 2008b).

The scientific approach to the phenomenon of heavy-metal tolerance has gone in two main directions: the knowledge of physiological processes and the knowledge of genecological and/or evolutive aspects.

1.1 The measurement and determination of tolerance

According to the definition we presented in previous paragraphs, tolerance to heavy metals is a complex trait defined by the interaction of genotypes and environment (Macnair 1993): different genotypes have a different response to increasing levels of metals (Fig. 1.1).

Although any species (or population) growing on a metalliferous substrate is considered as tolerant, an experimental demonstration of this tolerance (plants with different genotypes growing in a controlled environment with metals) is needed. The ideal test of tolerance should measure the effects of metal on fitness or

final yield on any species. Measuring this is difficult and laborious, so a growth parameter which is assumed to be correlated with fitness has to be used (Macnair 1993). The first and most commonly used parameter to characterise tolerance to heavy metals is the tolerance index (TI), which is calculated as:

TI = *Response to metal treatment / Response under control conditions*

Although the TI has received some criticism (Macnair 1990, Macnair 1993) it is still frequently employed, since it allows elimination of most of the variation in plant responses unrelated to metal treatment (Meyer *et al.* 2010).

Usually, the response is estimated by measuring root growth in a hydroponic culture (Wilkins 1978), but other variables such as shoot growth, leaf length or biomass are also used (reviewed in Köhl and Lösch 2004). As an alternative to these growth measures, several biomarkers, that is, biochemical, physiological or morphological changes owing to metal exposure have been used (plasmolysis capacity, pollen viability, seed germination, photosynthesis, respiration) and others have been tested in recent years (phytochelatin synthesis, ATP concentration, stress proteins…) (Köhl and Lösch 2004).

There are a wide variety of test designs for the determination of tolerance. Experiments can be developed sequentially (the same plant is first cultivated in control conditions and then subjected to a treatment with the metal) or in parallel (responses to control and to metal treatment are measured at the same time in different plants). It can be a single or multiple-concentration test, the latter using arithmetic or geometric series of metal concentrations (Schat and Ten Bookum 1992). Its duration may vary from around 2 days (short-term root elongation tests) to several weeks (long-term growth tests). In addition, the plants can be cultivated in hydroponic systems (with good reproducibility of nutrient and metal contents, accessibility to roots, easy harvesting of plants) or in solid media (whose cultivation conditions show a close resemblance to those in the field).

Given this wide diversity of approaches, as Macnair (1993) stated, "*the 'correct' test to use must be determined by the judgement of worker, based on experience of the species and metal under study*".

1.2 The physiology of tolerance

In one of the basic papers in this field, Baker (1981) ranked tolerant plants in three categories (accumulator, indicator and excluder) according to their patterns of response to increasing quantities of heavy metals in soils (Fig. 1.2).

Thus, an excluder shows low shoot levels of metals over a wide range of external concentrations up to a critical soil value above which the mechanism breaks down and unrestricted transport results. In contrast, accumulators concentrate metals in aerial plant parts from low or high soil levels, whereas 'indicators' show a proportional relationship between metal levels in

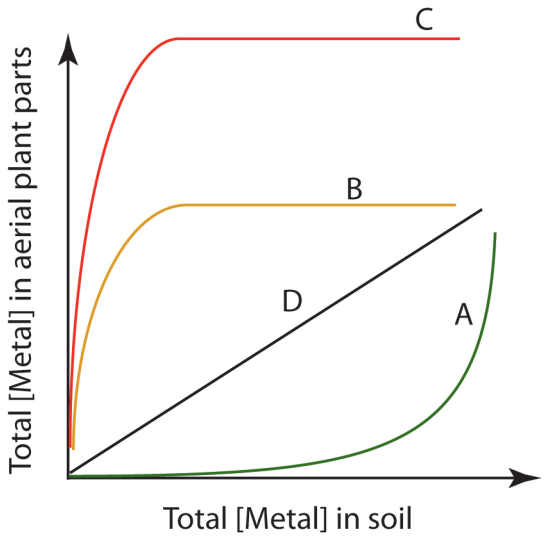

Figure 1.2: Classification of plant species on the basis of metal uptake. **A**: excluder. **B**: accumulator. **C**: hyperaccumulator. **D**: 'indicator'. Modified from Baker (1981) and Greger (2004).

the soil and accumulation in aerial plant parts. An additional category comprises the hyperaccumulators: plants that behave similarly to accumulators, but with a higher accumulation factor.

Several processes and mechanisms, both at the cellular and at whole organism level or even involving microorganisms from the rhizosphere, underlie these three general patterns.

At the cellular level, Hall (2002) cites the main mechanisms of tolerance (summarized in Fig. 1.3). Rather than developing proteins that can resist heavy metal effects, these mechanisms are aimed at preventing the build-up of toxic concentrations at the cytosol:

First, the role of rhizosphere organisms in tolerance has been demonstrated (reviewed in Kidd *et al.* 2009). Ectomycorrhizal fungi (which are exclusive to woody plants) develop a hyphae sheath that envelopes the root surface (and actually enters the intercellular spaces of the root cortex) and can reduce the inflow of metals to the plant host (point 1, Fig. 1.3). Moreover, the rhizosphere bacteria (mainly Plant Growth Promoting Rhizobacteria-PGPR) also provoke an increasing tolerance, given their role in the change in availability of metals in the rhizosphere and, especially, by secreting Plant Growth Promoting substances (referred as PGPs in point 2, Fig. 1.3).

Turning to the plant's own mechanisms, some of them act at the extracellular level. We refer to the binding of metals to the cell wall and, especially, immobilisation of metals outside the plant by binding to metal-chelating molecules exuded by roots (point 3, Fig. 1.3). E.g. *Thlaspi arvense* exposed to Ni increased the exudation of citrate and histidine by roots. These molecules form chelates with Ni and prevent its absorption by roots (Salt *et al.* 2000).

Other strategies to control the internal concentration of metals rely on the plasma membrane, mainly through reduction of the influx across the membrane by the modification of metal transporters (point 4, Fig. 1.3) e.g. arsenate tolerance in *Holcus lanatus* is related to the presence of an altered phosphate-arsenate uptake system (Meharg and Macnair 1990). The active efflux of metals through the plasma membrane to the apoplast has been proposed as another as another process involved in tolerance (point 5, Fig. 1.3). This

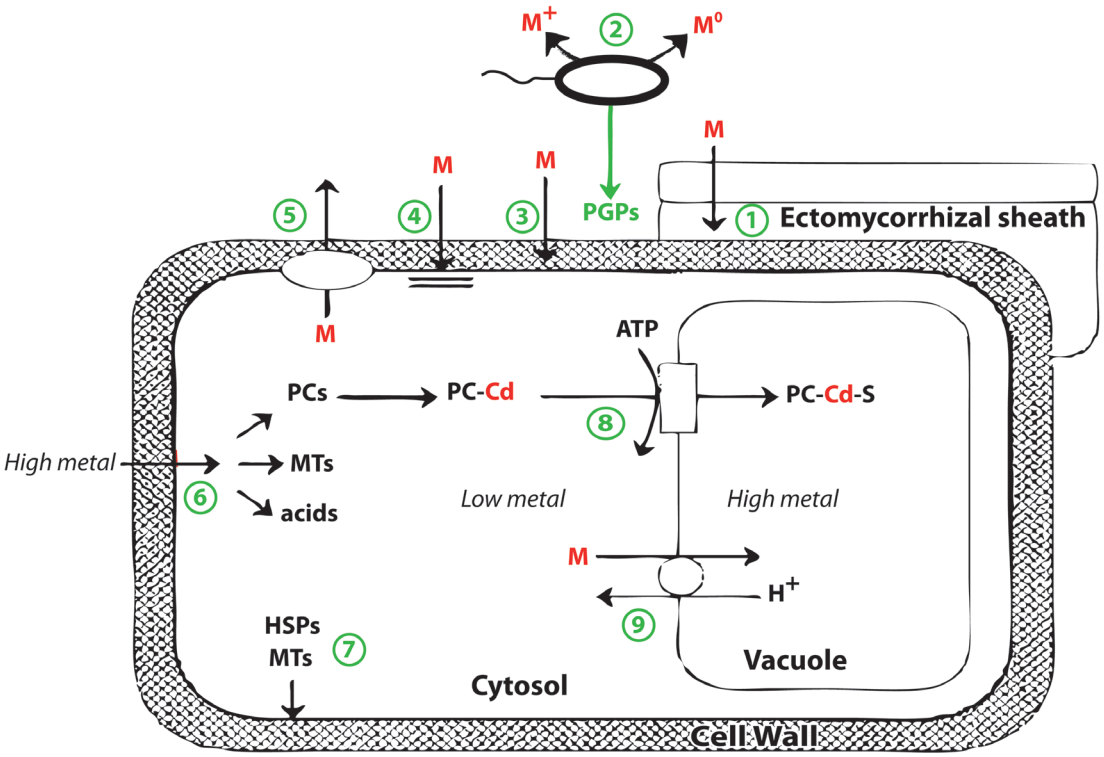

Figure 1.3: Summary of cellular mechanisms of metal tolerance in plants. **M**: metal atoms. **PCs**: phytochelatins. **MTs**: metallothioneins. **HSPs**: heat-shock proteins. **PGPs**: plant growth promoting substances. Bacteria are conventionally represented with a flagellum without any speculation on their biological status. Modified from Hall (2002).

mechanism is common in bacteria but with little direct evidence in plants (Hall 2002). For instance, metal transporter AtHMA4 has been described in *Arabidopsis thaliana*. This transporter may play a role in the translocation of Zn from root to shoot and it may be also involved in tolerance to Zn and Cd (Mills *et al.* 2005).

The remaining mechanisms are fully intracellular. They include, on one side. the chelation of metals by different organic ligands: aminoacids, organic acids and the cysteine-rich polypeptides Metallothioneins (MTs) and Phytochelatins (PCs) (point 6, Fig. 1.3). The formed chelates can then be removed from the cytosol by efflux outside the cell (e.g. complexation of Ni with histidine and transport to shoots) or pumped to the vacuole. This is the final step in the detoxification of Cd by PCs (point 8, Fig. 3).

However, free metals can be also sequestered in the vacuoles by different metal transporters in the tonoplast (point 9, Fig. 1.3). For instance, Zn tolerance in *Silene vulgaris* is mediated by theses uptake systems (Chardonnens *et al.* 1999).

The last mechanism of tolerance

to metals implies the repair and protection of plasma membranes under stressful conditions (point 7, Fig. 1.3). Heat Shock Proteins (HSPs) are the molecules mainly involved in this process, acting as chaperones in normal protein folding and assembly, as well as being involved in the protection and repair of proteins under metal stress.

Some of the mechanisms cited above are specific to the response against a particular metal, e.g. Cd detoxification by PCs; although the response to other trace metals may involve several mechanisms within the same cell or cells belonging to different tissues or organs. In this way, the redistribution of metals within the plant may play an important role in tolerance, depending on the general pattern of response (*sensu* Baker 1981).

Thus, in excluder species mechanisms to reduce the absorption of metals were predominant, and intracellular detoxification processes will occur mostly at the level of the roots. In contrast, in metal accumulators the main strategy is the transportation of metals to the aboveground plant parts and their accumulation in non-sensitive places. This mechanism is especially important in the extreme case of metal hyperaccumulating plants. For instance in *Stackhousia tryonii* (Ni hyperaccumulator) and *Thlaspi praecox* (Cd / Zn hyperaccumulator) metals are actively transported to aerial parts and accumulated preferentially in the vascular and epidermal leaf tissue, away from the photosynthetically active leaf tissues (Bhatia *et al.* 2004, Vogel-Mikuš *et al.* 2008, respectively).

1.3 Population genetics and evolution of tolerance

The study of population genetics has served to unveil the genetic bases of tolerance to heavy metals. In addition, metal-contaminated sites, either natural outcrops or mining deposits, may be considered as ecological islands which provide the opportunity to investigate the establishment and differentiation of plant populations under severe selection pressure (Lefèbvre and Vernet 1990).

Based on their presence or absence in metalliferous areas, plant species have been classified into three categories (Fig. 1.4). These categories are often related to the occurrence of genotypes tolerant to metals at the species level (Pollard *et al.* 2002):

- **Strict** (or obligate) **non-metallophytes** (species A in Fig. 1.4). Species restricted to soils with low metal concentrations, which is the case for great majority of plants.
- **Strict** (or obligate) **metallophytes**, also called eumetallophytes (species D in Fig. 1.4). These are taxa which grow exclusively on metalliferous soils. All the members of such taxa which have a tolerance to heavy metals are included Some species in this group are endemic to mine spoils or to serpentine soils, such as *Viola calaminaria* and *Alyssum serpyllifolium*, respectively.
- **Facultative metallophytes**, also called **pseudometallophytes** (species B and C in Fig. 1.4). Species with populations grow-

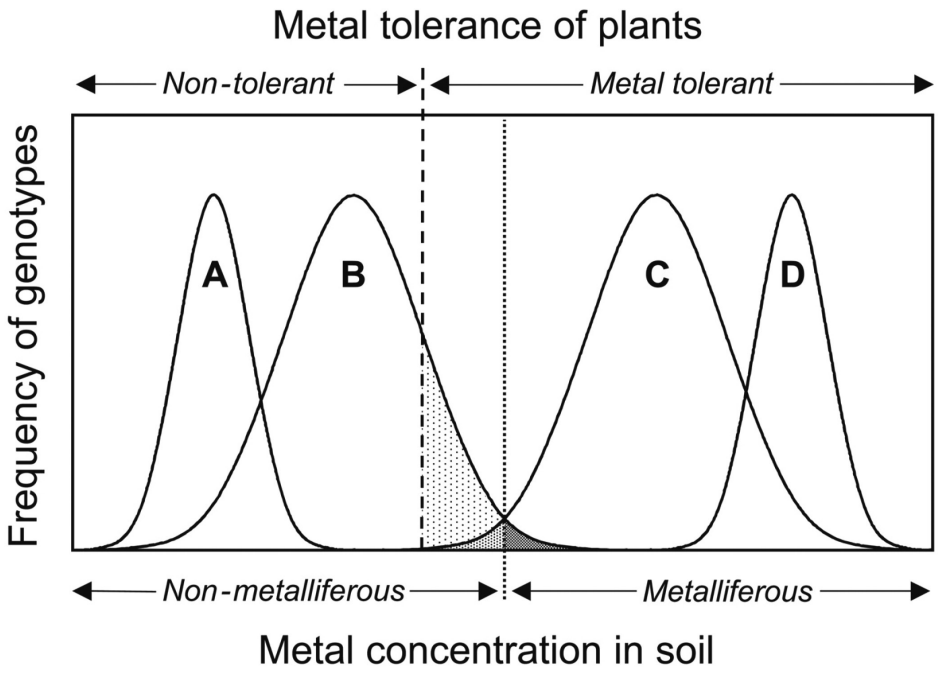

Figure 1.4: Patterns of plant metal tolerance (upper x-axis) and frequency distribution of plant genotypes on metal-containing soils exemplified for four contrasting species. **A**: strict non-metallophyte. **D**: obligate eumetallophyte. **B**: pseudometallophyte with ecotypic tolerance. **C**: pseudometallophyte with species-wide tolerance. Note that in some cases metal-tolerant genotypes are growing on non-metalliferous soils (shaded area). From Pollard *et al.* (2002).

ing on metalliferous and non-metalliferous soils belong to this group. Within the pseudometallophytes we can distinguish two sub-types: on one side, those species with **constitutive** (or **species-wide**) **tolerance**, that is, all genotypes are metal tolerant, even in those populations growing on soils with low metal contents (species C on Fig. 1.4); and on the other side, those species with **metal-tolerant races** or **ecotypes** that have evolved during the colonisation of metalliferous areas by populations where tolerant genotypes were at low frequencies (species B on Fig. 1.4) (Pollard *et al.* 2002).

To complete this relatively simple scheme, we add that several authors have stressed that plants which are, irrespective of their tolerance to metals, pre-adapted to any of the harsh edaphic conditions of metalliferous soils (nutrient shortage or imbalance, drought, high insolation, etc.) could colonize these soils more readily (Brady *et al.* 2005; and references therein), so, as Wu (1990) outlined, it is possible to colonize metalliferous soils without the evolution of tolerance.

In addition, it is important to consider the 'cost of tolerance', that is, the fact that metallicolous plants are at a

disadvantage in non-metalliferous soils. This physiological cost may explain the general absence of metallophytes in non-metalliferous substrates. Recent findings indicate that the cost of tolerance is not an effect of the tolerance genes, but is due to adaptation to the previously mentioned conditions or even to higher susceptibility to herbivores; it is therefore more precise to talk about the 'cost of adaptation to metalliferous environment' (Macnair *et al.* 2000, Dechamps *et al.* 2008 and references therein).

Given the within-species differences in tolerance to metals found in pseudometallophytes, these kinds of plants allow us to investigate the genetics and physiology of the differences between the tolerant and non-tolerant plants in a way that is generally not possible with the edaphic endemics (Macnair 1993). In addition, neighbouring metallicolous and non-metallicolous populations of pseudometallophytes are highly relevant models for the study of local adaptation in plants (Linhart and Grant 1996). Other interesting properties of pseudometallophytes are their high capacity to adapt to a wide range of adverse soil conditions, together with their usually higher bio-mass production and their good competitiveness in soils with moderate metal toxicity; they may therefore be useful for phytoremediation technologies (Poschenrieder *et al.* 2001).

On the basis of several experimental crosses between selected lines for tolerance and sensitivity to heavy metals within pseudometallophyte species, Macnair (1993) demonstrated that tolerance is a trait controlled by a small number (usually one) of major genes with main effects. This assertion is congruent with the results obtained by Wu *et al.* (1975), who indicated that evolution of tolerance can arise in a single generation.

Moreover, minor genes, present in greater numbers and less effective than major genes, have been discovered. These minor genes would be responsible for the variation in tolerance levels observed within and among metal-tolerant populations (Macnair *et al.* 2000). In addition, minor genes are hypostatic to major genes; that is, their effect on tolerance can be only observed if major genes are present.

Another interesting finding is that the co-tolerance (that is, the fact that tolerance to one metal confers a tolerance to other metals) has not been demonstrated; this fact contradicts the possible physiological role of nonspecific systems of metal tolerance, such as PCs, MTs and organic acids (Macnair *et al.* 2000).

The ease of growing, selecting and crossing herbaceous species in the laboratory has caused a certain bias in genetic studies of tolerance to herbs (Macnair 1993). Complementary to these laboratory studies, other lines of research employed population biology and genetics as tools for identifying evolutionary and genetic factors involved in tolerance (Pauwels *et al.* 2008a). Indeed, these population-based approaches are useful to study the origin and evolution of metal tolerance in woody pseudometallophyte species, with longer life cycles.

From a evolutionary point of view, researchers tried to address three major questions: i) Does colonisation of metalliferous areas imply a reduction in genetic diversity?, ii) Do metallicolous (M) populations share a common ancestry or are they the result of local colonization events? and iii) Have soil conditions promoted a significant genetic isolation between population types?

These three questions are ultimately related to the frequency of tolerance genes and also to the physiological cost of tolerance. If tolerance to heavy metals is not common in non-metallicolous (NM) populations of a certain pseudometallophyte species, it is expected that the metallicolous (M) population will be founded by a low number of mother plants. Thus, a founder effect (which implies a reduction of genetic diversity in M population and also a significante genetic differentiation between M and NM populations) may occur (Lefèbvre and Vernet 1990).

The different selection forces in metalliferous and non-metalliferous soils contribute to maintain the genetic differentiation between M and NM populations (Linhart and Grant 1996), whereas the gene flow between neighbouring M and NM populations may contribute towards homogenizing them. The differentiation between edaphic types can be promoted by the onset of barriers for gene flow between M and NM populations preventing the dilution of metal tolerance by pollen coming from non-tolerant populations (Lefèbvre and Vernet 1990). Some of these processes include increasing self-fertility in M populations, divergence in flowering time between M and NM populations, pollen-stigma incompatibiliy or hybrid sterility (Lefèbvre and Vernet 1990, Vekemans and Lefèbvre 1997).

In order to summarize the research carried out on the influence of the colonisation of metalliferous areas on the genetic structure of pseudometallophyte species, we have elaborated a table (Table 1.1), in which we present a review of papers on this topic arranged according to their year of publication. This table shows a progressive change in the molecular markers used in genetic studies (from isozymes and allozymes to DNA based markers), and also an increase in the number of populations and the geographic range considered.

These transitions are caused by the greater information provided by DNA compared to allozymes (markers very prone to homoplasy) and mainly for the need to separate the historical processes (phylogeography) from the selective processes really involved in the colonisation of M areas. As Staton *et al.* (2001) underline, the inference of the phylogeography of a species makes possible a better understanding of the effect of metal pollution on the genetic structure of populations, avoiding spurious correlations resulting from historical or demographic processes.

Among the DNA-based markers, those obtained from the chloroplast genomes (either chloroplast microsatellites –cpSSR- or chloroplast PCR-RFLP) are of special interest when studying colonisation patterns, and thus they have been widely used to infer the history of plant popula-

Table 1.1: Summary of the effects of the colonisation of metalliferous areas on the genetic diversity and/or the population genetic structure of different pseudometallophyte species.

Reference	Species	Markers	N of Pops			Colonisation of M areas		
			NM	M (s)	M (h)	Reduction Diversity	Genetic differentiation	Origin
Wu *et al.* (1975)	*Agrostis stolonifera*	Isozymes	2		9	No	n.e.	n.e.
Ducousso *et al.* (1990)	*Arrhenatherum elatius*	Allozymes	3		3	No	n.e.	n.e.
Westerbergh and Saura (1992)	*Silene dioica*	Isozymes	9	8		No	No	Multiple
Bush and Barret (1993)	*Deschampsia cespitosa*	Isozymes	8		10	Yes	Yes	Multiple
Vekemans and Lefèbvre (1997)	*Armeria maritima*	Allozymes	9	1	8	Yes	No	Multiple
Lehmann (1997)	*Calamagrostis epigejos*	Isozymes	2		2	No	n.e.	n.e.
Koch *et al.* (1998)	*Thlaspi caerulescens*	Isozymes	13		15	No	No	Multiple
Nordal *et al.* (1999)	*Lychnis alpina*	Isozymes	1		2	Yes	n.e.	Multiple
Mengoni *et al.* (2000)	*Silene paradoxa*	RAPD	1	5	2	No	Yes (mine vs. serpentines)	Multiple
Mengoni *et al.* (2001)†	*S. paradoxa*	cpSSR	1	5	2	Yes (mine pops)	No	Multiple
Nkongolo *et al.* (2001)	*D. cespitosa*	RAPD	2		7	n.e.	No	Multiple

n.e.: not estimated; † this paper analysed the same populations as Mengoni *et al.* (2000)

Table 1.1 (continued)

Reference	Species	Markers	N of Pops			Colonisation of M areas		
			NM	M (s)	M (h)	Reduction Diversity	Genetic differentiation	Origin
Nyberg Berglund and Westerbergh (2001)	*Cerastium alpinum*	Isozymes	19	12		No	No	Multiple
Dubois *et al.* (2003)	*T. caerulescens*	Allozymes	7		7	No	Yes (in one region)	n.e.
Pauwels *et al.* (2005)	*Arabidopsis halleri*	(cpDNA) PCR-RFLP	14		14	No	No	Multiple
Mengoni *et al.* (2006)	*Onosma echioides*	AFLP	3	5		No	No	n.e.
Baumbach and Hellwig (2007)	*A. maritima*	AFLP	12		10	No	No	Multiple
Deng *et al.* (2007)	*Sedum alfredii*	RAPD	2		5	Yes	Yes	n.e.
Jiménez-Ambriz *et al.* (2007)	*T. caerulescens*	nuSSR	3		3	No	No	n.e.
Pauwels *et al.* (2008)‡	*A. halleri*	(cpDNA) PCR-RFLP	50		14	No	No	Multiple

n.e.: not estimated; ‡ this paper used the same populations as Pauwels *et al.* (2005), as well as additional NM populations.

tions (Petit *et al.* 2003, Magri *et al.* 2007). CpDNA is generally maternally inherited in angiosperms, its dispersion is therefore carried out through seeds only. Thus, they are not influenced by pollen flow among M and NM populations.

In addition, the effective population size for haploid cpDNA is smaller than diploid nuclear genes, so the differentiation due to genetic drift may be stronger (Comes and Kadereit 1998) and phenomena like genetic bottlenecks may be more easily detected (Echt *et al.* 1998). For instance, cpSSR markers detected a reduction in genetic diversity within copper-mine populations of *Silene paradoxa* where RAPD markers failed (Mengoni *et al.* 2001).

Moreover, chloroplast microsatellites are considered to be neutral markers, that is, all their alleles (variants) have equal effects on the individual carrying them. Thus, cpSSRs provide a selection-independent framework which allows us to separate the effects of phylogeography from the effects of metal pollution.

As regards genetic diversity, a common trend across species is not found. In general, a founder effect has been detected in studies with populations on mining areas. This points to the effect of time together with the fact that most of the presented papers rely on markers related to nuclear DNA. Whereas the populations on mine tailings have supposedly been founded recently, the origin of populations on serpentine outcrops is generally older. A longer time implies a greater pollen flow from neighbouring non-metallicolous populations that could have increased genetic diversity and masked the putative founder effect. This is reflected by the aforementioned papers by Mengoni *et al.* (2000) and Mengoni *et al.* (2001) on *Silene paradoxa*.

In addition, several authors proposed that the increase in genetic diversity inferred in M populations of clonal grasses (e.g. *Arrhenatherum elatius* or *Calamagrostis epigejos*) is a sum of soil heterogeinity together with low intraspecific competition in polluted deposits, which allows the coexistence of a higher number of clones than in non-metalliferous soils (Ducousso *et al.* 1990, Lehmann 1997).

Only two pseudometallophytes have been studied with chloroplast markers: *Silene paradoxa* (Mengoni *et al.* 2001) and *Arabidopsis halleri* (Pauwels *et al.* 2005, Pauwels *et al.* 2008) with constrasting results. Pauwels *et al.* (2005) proposed that the colonisation of metal-polluted environments is associated with a genetic bottleneck in species with populational tolerance (as *S. paradoxa*), whereas in species with constitutive (or "specieswide") tolerance (such as *A. halleri*) the effect of a bottleneck may not be detected.

Genetic differentiation between population types has been detected in few works, and has mainly been related to geographic distances between M and NM populations than to a true effect of metals in soil (Dubois *et al.* 2003), or to a limited sampling that has made it impossible to distinguish between phylogeographic and selective effects (Bush and Barret 1993, Deng *et al.* 2007). As a rule, then, we may

conclude that the evolution of tolerance is not hampered by the existence of gene flow among populations, possibly due to high selective pressures in metalliferous soils (Vekemans and Lefèbvre 1997). This is clearly congruent with the fact that M populations have multiple origins, that is, they have originated locally from NM populations that colonised metalliferous areas. In addition, M populations from a certain area are more genetically similar to neighbouring NM populations than to distant M ones.

Given the fact that variations in tolerance and accumulation capacity are genetically controlled, metallicolous populations with independent origins might show different patterns of response to heavy metals, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Silene armeria* (Llugany *et al.* 2003), *Cerastium alpinum* (Nyberg Berglund *et al.* 2003) and *Thlaspi caerulescens* (Assunção *et al.* 2003).

Over the last two decades, the field of population genomics has developed with the aim of identifying loci causing adaptive differences in natural populations. Population genomics is based on the use of genome scans; that is, the screening of genome-wide patterns of DNA polymorphism to detect 'outlier loci' which are subjected to positive directional selection (Storz 2005, and references therein).

Based on Amplified Fragment Length Polymorphism (AFLP, Vos *et al.* 1995), a kind of DNA-marker which can be applied to any organism without previous knowledge of sequences, the genome scans have been applied to non-model organisms (Bonin *et al.* 2007). In the case of plants, genome scans with AFLP have been used for taxonomic purposes (Scotii-Saintagne *et al.* 2004, Savolainen *et al.* 2006), or to detect loci potentially involved in adaptation to different ecological conditions (Namroud *et al.* 2008, Parisod and Christin 2008, Poncet *et al.* 2010). Moreover, genome scans may play a promising role in the studies of adaptation and evolution of tolerance to heavy metals; for instance, Meyer *et al.* (2009) have inferred loci putatively involved in tolerance to heavy metals in M and NM populations of the pseudometallophyte *Arabidopsis halleri*.

1.4 *Cistus ladanifer* L., an interesting pseudometallophyte

The gum rockrose ('esteva' in Galician-Portuguese or 'jara pringosa' in Castillian-Spanish) (*Cistus ladanifer* L.; Fam. Cistaceae) is a woody shrub from the Western Mediterranean Area (from Southern France to the North of Morocco and Algeria) (Demoly and Montserrat 1993).

1.4.1 Taxonomy, description, breeding system and seed dispersal

An adult *C. ladanifer* plant may reach a height of 2m, with dense root and shoot systems (Martín Bolaños and Guinea López 1949). It has lanceolate green leaves with a glabrous upper surface, whereas the back is covered with a white tomentum. Leaves are presented in a decussate arrangement, and welded at their base. The morphology of leaves is used

as a characteristic for the identification of its three recognized subspecies: subspp. *africanus*, *ladanifer* and *sulcatus* (Demoly and Montserrat, 1993). Subsp. *africanus* has petiolate leaves whereas those of subspp. *ladanifer* and *sulcatus* are sessile. Moreover, in subsp. *sulcatus* (originally described as *Cistus palhinhae*) the leaf surface is split by well marked veins.

Subsp. *ladanifer* is primarily distributed in the Iberian Peninsula, northern Africa and France (where it is considered an introduced species); subsp. *sulcatus* is endemic to south-western Portugal (Algarve region); and subsp. *africanus* is present in southern Spain (Cádiz, Málaga), but more commonly found in northern Africa (Demoly and Montserrat 1993). Guzmán and Vargas (2009) have dated the origin of *C. ladanifer* and divergence of its different subspecies in the Upper Pleistocene.

Its flowers are large (55 to 70 mm diameter) and solitary, with 3 sepals and 5 white petals (var. *albiflorus*) which also may have a red to maroon spot at the base (var. *maculatus*). Each flower has a sessile stigma and a great number of stamens which may produce more than 700 000 pollen grains per flower (Talavera *et al.* 1993).

Pollination is entomophyllous (mainly Diptera, Hymenoptera and Coleoptera). A gametophytic mechanism of incompatibility exists, so *Cistus ladanifer* is an obligate outcrosser (Talavera et al. 1993). This fact, together with the short distances of pollen dispersal (only a few meters) results in reduced reproductive output in isolated plants (Metcalfe and Kunin 2006).

The fruit is a loculicide capsule with 5-7 to 12 locules. A single capsule may contain around 1,000 seeds, so it is estimated that a single adult *C. ladanifer* plant may produce more than 158,000 seeds each year. Seed release starts in mid-summer and continues for 8 to 10 months (Bastida and Talavera 2002). Seed dispersal is mainly barochorous, and more than 80% of seeds fall beneath the mother-plant canopy (Bastida and Talavera 2002), however different granivorous ants of genus *Goniomma* and the red-deer (*Cervus elaphus*), which feed on seeds and fruits respectively, may play an important role in seed dispersal over longer distances (Bastida and Talavera 2002; Malo and Suárez 1998).

1.4.2 Competitive traits

Cistus ladanifer populations constitute early successional stages adapted to disturbances operating in Mediterranean ecosystems, especially fire: its seeds maintain viability for several years (3-year-old seeds have a germination rate as high as 80%; *personal observation, C. Quintela-Sabarís*) and have a physical dormancy mechanism that can be interrupted by fire, high temperatures (Pérez-García 1997), smoke and nitrogenous salts (Pérez-Fernández and Rodríguez-Echeverría 2003).

The post-fire recovery of plants is accomplished by massive seedling emergence during the first post-fire year (Ferrandis *et al.* 1999), which allows rapid regeneration of original populations (2 years after experimental burning, *C. ladanifer*

covers 40% of the original area; Calvo *et al.* 2005).

Moreover, this species is well adapted to water and light stress. It develops dense shallow root systems that favour water uptake in transient wet periods (Martín Bolaños and Guinea López 1949). In addition, it is semi-deciduous, retaining small-sized potentially active leaves through the summer drought (Núñez-Olivera *et al.* 1996). Leaves are also photoprotected by the exudation of a fragrant, sticky resin (labdanum) which increases during summer (Chaves *et al.* 1993). Due to its content in flavonoids and other phenolyc compounds, the secretion of labdanum has other beneficial effects for *C. ladanifer* plants: it is an allelopathic compound that inhibits the germination of other plants (Herranz *et al.* 2006) and it provides a defence against herbivores eating *C. ladanifer* leaves through impairment of mouth skeletal muscle relaxation (Sosa *et al.* 2004).

1.4.3 Relation to soils

Cistus ladanifer is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). However, distribution models support its tolerance to calcareous soils (Gastón *et al.* 2009) and subsp. *sulcatus* is even restricted to limestone-derived soils on coasts from SW Portugal.

Its ability to develop in nutrient-poor areas may have been favoured by the establishment of symbioses with microorganisms in roots. Several bacteria strains with phosphate solubilisation or siderophore production (one of them identified as Plant Growth Promoting Rhizobacteria - PGPR) have been isolated from *C. ladanifer* roots (Ramos Solano *et al.* 2006). In addition, more than 30 fungal species have been recorded as forming symbiotic relations (ectomycorrhiza) with *C. ladanifer* in the literature (Comandini *et al.* 2006).

But most important among all these interesting features is the fact that ***C. ladanifer* is a pseudometallophyte**. Subspecies *ladanifer* and *africanus* have successfully established populations on serpentine areas and on mine tailings, where in some cases they are the dominant species.

In table 1.2 we summarize the papers reporting the occurrence of gum rockrose on metalliferous substrates, which also quantify the contents of diverse heavy metals in different plant organs collected in the field. In some of these papers (Alvarenga *et al.* 2004, Pratas *et al.* 2005, Murciego Murciego *et al.* 2006, de la Fuente *et al.* 2010), *C. ladanifer* has been described as an indicator, or even an accumulator, of As, Sb and Zn, and Mn, Sb and W, respectively.

These references provide useful information about gum rockrose and metals. However, all are local or regional-based reports, using different methodologies for the sampling and quantification of metals, so it is difficult to make comparisons between them.

To our knowledge, only three papers assess the tolerance of *C. ladanifer* to

Table 1.2: Heavy metal contents (as $mg.kg^{-1}$) quantified in different organs of field-collected *Cistus ladanifer* plants. This table includes the bibliographic reference (in alphabetical order), the area of study and the analysed plant organs. Data indicate mean values, except those data in **bold type**, which refer to maximum contents. Empty cells indicate elements not quantified in the study.

Reference	Area of study	Plant organs	Ag	Al	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	Se	Sn	W	Zn
Alados *et al.* (1999)	Ultramafic area in Málaga (S Spain)	leaves										10.1						
Alvarenga *et al.* (2004)	Pyrite mine tailing (Alentejo, S Portugal)	leaves							**29.7**		**1399**		**25.8**					**314**
		roots							**80.6**		**357**		**29.1**					**92.6**
Ater *et al.* (2000)	Bni Bouchra ultramafic area (N Morocco)	leaves										98.0						
Batista (2003)	Neves Corvo mining area (Alentejo, S Portugal)	leaves	**0.56**	**4315**	**48.2**		**11.9**	**10.1**	**591.5**		**2852**	**20.2**	**24.1**	**8.5**		**20.2**	**0.2**	**177**
		roots	**0.04**	**1788**	**6.7**		**1.4**	**55.0**	**176**		**1233**	**19.5**	**7.2**	**1.13**		**82.7**	**0.1**	**70.7**
Casado *et al.* (2007)	Losacio and Cogollas old mines (Zamora, N Spain)	aerial parts			**1.8**									**0.71**				
Chopin and Alloway (2007)	Mining area Iberian Pyrite Belt (SW Spain)	aerial parts			**30**				**460**				**237**					**729**
Díez Lázaro *et al.* (2006)	Ultramafic outcrops and surrounding area in Trás-os-Montes (NE of Portugal)	leaves						**128**	**15.0**		**2000**	**50.0**						**300**
		stems						**26.0**	**11.0**		**467**	**50.0**						**500**
		roots						**17.0**	**15.0**		**350**	**75.0**						**140**

Table 1.2 (continued)

Reference	Area of study	Plant organs	Ag	Al	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	Se	Sn	W	Zn
Freitas *et al.* (2004a)	Abandoned mine within ophiolitic complex (Trás-os-Montes, NE of Portugal)	leaves					1.7	1.9	3.5		60.7	18.9	1.3					36.2
		fruits					0.7	0.4	5.3		14.9	10.8	0.7					25.6
		roots					6.3	40.8	19.4		100.3	99.9	4.1					56.2
Freitas *et al.* (2004b)	Abandoned Copper mine in SE Portugal	leaves	0.1		2.1				13.4			4.6	11.0					170.1
		twigs	0.2		1.8				10.0			3.7	21.4					80.9
De la Fuente *et al.* (2010)	Rio Tinto basin (SW Spain)	aerial parts			9.45				26.3		664	3.51	10.6					113
Millán *et al.* (2006)	Almadén mining area (C Spain)	aerial parts								**5.1**								
Murciego Murciego *et al.* (2007)	Sb mining areas (Extremadura, SW Spain)	leaves												96.0				
Pratas (1996)	Borralhal mine (C Portugal)	leaves			**1.36**				**17.65**				**6.32**					**144.7**
Pratas *et al.* (2005)	Two abandoned mines in C Portugal	leaves			**2.77**									**10.6**			**30.7**	
		twigs			**2.38**									**29.3**			**3.55**	
Reglero *et al.* (2008)	Old lead mining area (C Spain)	leaves			0.25	0.49			12.5				13.6		0.37			98.5
		flowers			*nd*	0.16			10.8				2.60		0.17			36.9
Santos *et al.* (2009)	Abandoned Copper mine in SE Portugal	young leaves			1.2				8.1				47.4					127.0
Soldevilla *et al.* (1992)	Rio Tinto pyrite mine complex (SW Spain)	leaves		282		1.8			42		691		8					127

nd: not detected

heavy metals, each of them using different approaches.

- Alados *et al.* (1999), using developmental stability analysis demonstrated the adaptation of *C. ladanifer* to serpentine soils in Málaga (S of Spain). These authors also introduce the hypothesis that the low Ca^{2+} requirements of this species could be an advantage in the colonisation of serpentines. Along these lines, Ater *et al.* (2000) quantified a high Mg/Ca ratio in leaves of *C. ladanifer* growing in serpentines from N of Morocco.
- Kidd *et al.* (2004) subjected plantlets of 5 populations growing on metalliferous and non-metalliferous soils from NE Portugal to experiments of tolerance to Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn in hydroponic culture. They observed population-specific patterns of tolerance and accumulation and inferred that even plants from non-metallicolous populations showed relative tolerance to metals. We analysed 4 of these populations with RAPD markers and we found similar levels of genetic diversity within metallicolous and non-metallicolous populations, in addition we found a significant differentiation between the groups of metallicolous and non-metallicolous populations. However, we were not able to determine if the origin of this structuring was related to phylogeography or to the colonisation of metalliferous areas (Quintela-Sabarís *et al.* 2005).
- Santos *et al.* (2009) sought differential activity of antioxidative enzymes in *C. ladanifer* plants from an abandoned mine area in SE Portugal, but they did not find any variations related specifically to metals.

In a highly interesting PhD Thesis, Díez-Lázaro (2008) dealt with the optimisation of the use of *Cistus ladanifer* for phytoremediation procedures. He found that the addition of fertilizers and the acidification of soil improve the growth and the extraction of Mn and Zn by C. ladanifer plants from NE Portugal. In addition, he stated that gum rockrose could be readily used for the phytoextraction of Zn in soils with low to medium contents of this metal.

Finally, the beneficial effect of this plant is underlined by Simões *et al.* (2009), who found that it can produce up to 4,600 kg of dry matter ha^{-1} $year^{-1}$ of litterfall, which improves soil quality and may promote vegetation regeneration by facilitating the invasion of more demanding species.

In summary, *C. ladanifer* possesses a series of interesting traits that make it especially useful for the recovery of degraded areas in the Mediterranean region. In addition, it is a native species to this biodiversity-rich region, and therefore its use would not produce the detrimental effects on the surrounding ecosystems produced by alien and invasive species (Méndez and Maier, 2008; and references therein).

1.5 Objectives

According to the items presented above, *Cistus ladanifer* seems to be a promising species in phytoremediation procedures in the Mediterranean region and is also an interesting model species for

the study of the process of colonisation of metalliferous areas by plants.

Within this framework, and with the aim of improving the knowledge about *Cistus ladanifer* and its relationships with metals, we have developed a series of investigations using populations sampled from nearly the entire distribution area, in order to deal with the following topics:

- In order to infer the effects of the metals on the genetics of the species, it is first of all essential to understand the interplay of processes that creates its phylogeography, or genetic landscape. Using neutral maternally-inherited markers (cpSSRs) we inferred the phylogeography of *Cistus ladanifer* (**Chapter 2**).

- Then we integrated the soil type (metalliferous or non-metalliferous) and the phylogeographic information in a population-genetics approach to the tolerance of *Cistus ladanifer* to metals: are the metallicolous populations mono- or polyphyletic? Is the colonisation of metalliferous areas accompanied by a reduction in genetic diversity? (**Chapter 3**).

- A species range is not homogeneous, but it is often subdivided in into genetic subgroups. If metallicolous populations have evolved independently within different subgroups, it is interesting to infer whether the parallel evolution resulted in similar or different strategies of tolerance (exclusion, accumulation?). We assessed this theme, within the framework provided by cpSSR, through the analysis of field-collected soils and *C. ladanifer* leaves (**Chapter 4**) and through hydroponic-based experiments of tolerance to Co, Ni and Zn (**Chapter 5**).

- As a basis for future research, the identification of markers potentially linked to tolerance to metalliferous soils is especially needed in non-model plants such as *C. ladanifer*. We address this topic applying generalized estimating equations (GEE) to AFLP markers and data of total metal contents in soils (**Chapter 6**). We also compared the information on population genetics provided by AFLP markers (from nuclear DNA) and cpSSRs.

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Chapter 2

Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region

This chapter reproduces the published paper: Quintela-Sabarís C, Vendramin GG, Castro-Fernández D, Fraga MI (2011) Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region. Plant Biology 13:391-400

Previous page: General view of the vegetation in the Despeñaperros Gorge area (Sierra Morena, Jaén province, S of Spain). Here, *Cistus ladanifer* subsp. *ladanifer* coexists with *Quercus ilex* and *Juniperus oxycedrus* plants. (Photo: C. Quintela-Sabarís)

CHAPTER 2

Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region

Keywords: Chloroplast microsatellites; glacial refugia; Iberian Peninsula; phylogeography; population genetics; Strait of Gibraltar.

ABSTRACT

This study investigated the phylogeographic structure of *Cistus ladanifer*, in order to locate its Quaternary refugia, reconstruct its recolonisation patterns and assess the role of geographical features (mountain ranges, rivers and the Strait of Gibraltar) as barriers to its seed flow and expansion through the Western Mediterranean.

Thirty-eight populations were screened for length variation of polymorphic chloroplast simple sequence repeats (cpSSRs). Statistical analyses included estimation of haplotypic diversity, hierarchical analysis of molecular variation (AMOVA) and fixation indices. Mantel tests, SAMOVA and BARRIER analyses were applied to evaluate the geographical partitioning of genetic diversity across the entire species range.

Pollen data from bibliography were used to complement molecular inferences Chlorotype diversity within populations was similar throughout the natural range of *C. ladanifer* (mean haplotypic diversity = 0.32). High differentiation among populations was estimated (GST = 0.60).

Our data suggest that the barriers of the Strait of Gibraltar and the Betic ranges may have favoured the divergence during glacial periods of four different lineages of populations inferred with SAMOVA. The main northward colonisation of in the Iberian Peninsula occurred from refugia in southwest Iberia. This process may have been influenced by human activities (forest clearance, livestock grazing and even commerce) in the Iberian Peninsula. In contrast, populations in the Betic area have conserved a specific haplotype.

2.1 Introduction

The Iberian Peninsula is considered one of the most important Pleistocene glacial refugia in Europe. It is a large area (about 580,000 km^2) with a high degree of physiographic, climatic and geologic variability, which has favoured the occurrence of multiple isolated glacial refugia (Gómez and Lunt 2007). During the last glacial maximum, Mediterranean taxa were restricted to southern and southeast edges of the Iberian Peninsula and to large areas of North Africa (Carrión *et al.* 2003, López de Heredia *et al.* 2007). These taxa expanded from the refugia during interglacial periods. According to the 'leading edge' hypothesis, the post-glacial expansion of species range mainly involved populations from northern edges of the refugia, which spread rapidly (by means of long-distance dispersal events) into new territories and significantly precluded the northward expansion of later-arriving lineages (Hewitt 2001). This hypothesis also predicts a significant geographical separation of lineages expanding from different refugia and, because of multiple successive founder events, a northward decrease in genetic diversity.

In addition to the geographical position of the refugia, the presence of

barriers that preclude or limit expansion is another important factor that plays a role in shaping the present genetic structure of plant populations (Taberlet *et al.* 1998): there are several mountain ranges in the Iberian Peninsula with an east–west orientation. These orographic barriers have, on the one hand, enabled survival of populations by altitudinal shifts as a consequence of climatic changes (e.g., *Pinus sylvestris*; Sinclair *et al.* 1999) but, on the other hand, might represent effective barriers to gene flow and recolonisation along the north–south axis. As well as mountain ranges, the Strait of Gibraltar could have influenced the genetic structure of plant species that survived in southern Spain during the Pleistocene ice ages successive range contractions and expansions. However, the role of this strait as a biogeographic barrier differs among plant species according to Rodríguez-Sánchez *et al.* (2008). These authors observed an absence of significant differentiation among populations separated by the Strait of Gibraltar in pioneer species with high seed dispersal and establishment potential. Human activities represent an additional factor, which, in combination with geological history and climate changes, have shaped plant diversity in the Mediterranean region (Thompson 2005). Human impact through forest clearing, use of fire and grazing caused retraction of some species to isolated patches, but also created new opportunities for colonisation and the spread of other species (e.g., *Cistus ladanifer*).

Cistus ladanifer L. (gum rockrose) is a woody, semi-deciduous shrub that grows in a wide range of habitats in the Western Mediterranean (South of France, Iberian Peninsula and northern Algeria and Morocco) (Demoly and Montserrat 1993), where it constitutes a major component of the landscape. Populations of this species represent early successional stages adapted to disturbances in Mediterranean ecosystems, particularly fire (Bastida and Talavera 2002). *C. ladanifer* is a major element of the dehesas and montados, forests of *Quercus ilex* and *Q. suber* in southwest Spain and south Portugal, partially cleared to enable extensive livestock grazing. Its role as a coloniser of disturbed areas and its distribution on both sides of the Strait of Gibraltar and throughout the Iberian Peninsula, make this an interesting species for phylogeographic and local differentiation studies. The flowers produce high quantities of pollen (mean 631,509 grains / flower; Talavera *et al.* 1993), which is mainly dispersed over short distances (Metcalfe and Kunin 2006). However, only well conserved *C. ladanifer* pollen grains can be easily distinguished from those of other *Cistus* species, so paleoenvironmental reconstructions generally use the '*Cistus* type' category, where pollen from different *Cistus* species and even other Cistaceae genera (such as *Helianthemum* or *Xolantha*) are considered together. This fact makes it difficult to reconstruct the expansion of *C. ladanifer* based solely on pollen data, particularly in the Iberian Peninsula and Morocco, where 12 *Cistus* species occur, each with different ecological requirements (Demoly and Montserrat 1993, Soriano 2002).

The use of molecular markers has enabled clarification of the post-glacial migration of plant species for which there are very limited fossil pollen records, such as *Ilex aquifolium* and *Hedera* species (Grivet and Petit 2002, Rendell and Ennos 2003). Chloroplast DNA (cpDNA) is inherited through the maternal line in *C. ladanifer* (Guzmán and Vargas 2009), and thus reflects only the effect of seed flow and seed dispersal. In addition, the effective population size for haploid cpDNA is smaller than for diploid nuclear genes, so that differentiation through genetic drift may be stronger (Comes and Kadereit 1998). These characteristics justify the wide use of cpDNA to infer the history of plant populations (Petit *et al.* 2003, Magri *et al.* 2007, Petit and Vendramin 2007). We analysed chloroplast microsatellite (cpSSR) variation in *C. ladanifer* throughout its distribution range in order to: (i) locate its putative Quaternary refugia and reconstruct its recolonisation patterns in the highly heterogeneous Iberian Peninsula; and (ii) assess the role of geographic features such as mountain ranges, rivers and the Strait of Gibraltar as barriers to seed flow and expansion of the species. Whenever possible, molecular evidence is complemented with pollen data from bibliographic references.

2.2 Material and Methods

2.2.1 The Species

Cistus ladanifer is an entomophyllous, obligatory outcrossing species, with a gametophytic mechanism of self-incompatibility (Talavera *et al.* 1993). An individual plant can produce more than 100,000 small, long-lived seeds each year (Bastida and Talavera 2002), which mainly fall beneath the mother plant canopy (Malo and Suárez 1998, Bastida and Talavera 2002). The seeds have a physical dormancy mechanism that can be interrupted by fire, high temperatures (Pérez-García 1997) and smoke and nitrogenous salts (Pérez-Fernández and Rodrıíguez-Echeverría 2003). The post-fire recovery of plants in the Cistaceae is accomplished by massive seedling emergence during the first post-fire year (Ferrandis *et al.* 1999), which allows rapid regeneration of original populations (2 years after experimental burning, *C. ladanifer* covers 40% of the original area; Calvo *et al.* 2005). Although the main dispersal strategy of this species is barochory, different authors have described endozoochory by red deer (Malo and Suárez 1998) and sheep (Manzano *et al.* 2005) as other mechanisms of long-distance seed dispersal.

Three *C. ladanifer* subspecies (*africanus*, *ladanifer* and *sulcatus*) have been described based on leaf traits (Demoly and Montserrat 1993). Subspecies *ladanifer* and *africanus* are present in the Iberian Peninsula and in North Africa, whereas subspecies *sulcatus* is restricted to limestone-derived soils on the southwest Iberian coast. A recent work (Guzmán and Vargas 2009) has dated the origin of C. ladanifer and divergence of its different subspecies in the Upper Pleistocene.

2.2.2 Plant sampling

Thirty-eight *C. ladanifer* populations, covering almost the entire natural range of the species and its three subspecies, were sampled (Table 2.1). A longitudinal transect was established at each population. Ten plants separated by at least 5 m were selected along the transect and their ripe fruits were collected. Seeds were sown in Petri dishes and grown to seedling stage in the laboratory. One seedling per mother plant was selected for the subsequent analyses. Young plants were frozen in liquid nitrogen and conserved at)20 C until DNA extraction. In three populations (ESA, FVI and FVII), DNA was extracted from field-collected mature leaves from at least nine mother plants.

2.2.3 DNA extraction

DNA was extracted from 100 mg of frozen leaves with a Dneasy® Plant Mini Kit (Qiagen, Hilda, Germany), following the manufacturer's instructions. In several cases, an additional wash with 500 µl of absolute ethanol was necessary to remove secondary compounds from the DNA extracts.

2.2.4 Microsatellite analysis

In an initial screening, universal cpSSR primers ccmp1 to ccmp10 (Weising and Gardner 1999) and Fagaceae cpSSR primers cmcs 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13 and 14 (Sebastiani *et al.* 2004) were tested on a subset of 30 samples from six geographically distant populations. Only primers ccmp1, ccmp2, ccmp3, ccmp5, ccmp10 and cmcs1 yielded consistent amplifications, of which ccmp1, ccmp5, ccmp10 and cmcs1 were monomorphic. The two polymorphic cpSSRs were then used to amplify all samples of the 38 populations. Amplification reactions were performed in a total volume of 12.5 µl containing 0.5 U GoTaq®DNA Polymerase (Promega, Madison, WI, USA), under standard reaction conditions. DNA was amplified under the following thermal profile: one denaturation cycle of 4 min at 95 C, followed by 25 cycles each consisting of 95 C for 30 s, 50 C for 30 s, 72 C for 30 s, with a final extension step at 72 C for 8 min. PCR products were loaded onto a 96 capillary automatic sequencer MegaBACE 1000 (GE Healthcare, Uppsala, Sweden). MegaBACE ET400 (GE Healthcare) was used as size standard. Fragment lengths were determined with the MegaBACE Fragment Profiler software, version 1.2 (GE Healthcare).

Table 2.1: Populations included in this work. The country of origin is shown next to population name. *FR*: France; *MO*: Morocco; *PT*: Portugal; *SP*: Spain. *Subsp*: *C. ladanifer* subspecies. *afr*: subsp. *africanus*. *lad*: subsp. *ladanifer*. *sul*: subsp. *sulcatus*. Latitude and longitude are shown as decimal degrees; *N.S.*: number of plants surveyed in each population. The last three columns indicate different indices of within-population haplotypic diversity: N_e: effective number of haplotypes; H_E: Nei's (1987) haplotypic diversity; D^2_{SH}: average genetic distances between individuals (Vendramin *et al.* 1998); *Mean*: mean ± standard deviation values.

Population/Country	Code	Subsp	Lat	Long	N.S.	N_e	H_E	D^2_{SH}
Ketama /MO	MKE	afr	34.95º N	4.64º W	10	1.52	0.38	0.22
Bni Hadifa /MO	MBH	afr	35.02º N	4.17º W	10	1.22	0.20	0.10
Bab Tazaa /MO	MBT	lad	35.09º N	5.24º W	10	1.22	0.20	0.10
El Jebha /MO	MEJ	afr	35.18º N	4.64º W	10	1.00	0.00	0.00
Bni Bouchra/MO	MSI	afr	35.30º N	4.90º W	10	1.92	0.53	0.27
Bni Bouchra /MO	MSII	afr	35.30º N	4.89º W	12	2.77	0.70	0.58
Tanger /MO	MTA	afr	35.78º N	5.93º W	10	1.00	0.00	0.00
Almodóvar /SP	EAL	lad	36.16º N	5.65º W	10	1.00	0.00	0.00
Benalup de Sidonia /SP	EBE	afr	36.32º N	5.73º W	10	2.38	0.64	0.76
Sierra Bermeja /SP	ESB	lad	36.48º N	5.18º W	10	1.00	0.00	0.00
Sierra Palmitera /SP	ESP	lad	36.60º N	5.07º W	9	1.59	0.42	0.50
Tolox /SP	ETO	lad	36.68º N	4.93º W	10	1.22	0.20	0.10
Grazalema /SP	EGR	lad	36.78º N	5.27º W	10	1.00	0.00	0.00
Sierra de Aguas /SP	EAC	lad	36.84º N	4.79º W	10	1.00	0.00	0.00
Sierra Alhamilla /SP	ESA	lad	36.99º N	2.30º W	10	1.00	0.00	0.00
São Vicente cape /PT	PSV	sul	37.03º N	8.98º W	10	1.22	0.20	0.10
Burgau /PT	PBU	sul	37.07º N	8.78º W	9	1.53	0.39	0.19
Mazagón /SP	EMA	lad	37.15º N	6.84º W	10	2.27	0.62	1.78
Corte Figueira /PT	PCF	lad	37.39º N	8.03º W	9	1.53	0.39	0.19
Aljustrel /PT	PAL	lad	37.88º N	8.18º W	9	2.31	0.64	0.53
Cardeña /SP	ECA	lad	38.28º N	4.36º W	10	2.78	0.71	0.89
Despeñaperros /SP	EDE	lad	38.39º N	3.51º W	10	2.38	0.64	0.46
El Guijo /SP	EGJ	lad	38.52º N	4.77º W	10	1.72	0.47	0.23
La Codosera /SP	ECO	lad	39.19º N	7.08º W	10	2.00	0.56	0.28
Valdecaballeros /SP	EVC	lad	39.33º N	5.34º W	10	2.78	0.71	0.71
Martinchel /PT	PMA	lad	39.52º N	8.29º W	3	1.80	0.67	5.33
Vela /PT	PVE	lad	40.44º N	7.29º W	9	1.00	0.00	0.00
Ciudad Rodrigo /SP	ECR	lad	40.63º N	6.49º W	10	1.47	0.36	0.18
Sierra Guadarrama /SP	EGU	lad	40.68º N	4.10º W	10	1.22	0.20	0.10
Fuente Saúco /SP	EFS	lad	41.19º N	5.51º W	10	2.38	0.64	0.46
Macedo dos Cavaleiros /PT	PMC	lad	41.52º N	6.82º W	10	1.00	0.00	0.00
Ricobayo /SP	ERB	lad	41.70º N	5.81º W	10	1.92	0.53	0.27
Samil /PT	PSA	lad	41.78º N	6.75º W	7	1.00	0.00	0.00
Bragança /PT	PBR	lad	41.85º N	6.87º W	10	1.92	0.53	0.27
Monte Furado /SP	EMF	lad	42.39º N	7.20º W	10	1.00	0.00	0.00
Bárcena /SP	EBA	lad	42.57º N	6.56º W	10	1.85	0.51	0.72
La Bouverie /FR	FVI	lad	43.48º N	6.66º E	9	1.00	0.00	0.00
La Bouverie /FR	FVII	lad	43.49º N	6.66º E	9	1.00	0.00	0.00
				Mean ± SD		1.58 ± 0.59	0.32 ± 0.27	0.40 ± 0.90

2.2.5 Data analysis

Genetic diversity within populations was assessed as the effective number of haplotypes ($Ne = 1/\Sigma p_i^2$), the haplotypic diversity ($H_E = [n/(n-1)][1-\Sigma p_i^2]$, where n is the number of individuals analysed in a population and p_i is the frequency of the ith haplotype in a population; Nei 1987) and the D^2_{SH} measure, as defined by Vendramin *et al.* (1998). The latter measure takes into account the difference in number of repeats among the different cpDNA haplotypes considered. The correlation between population genetic diversity parameters and latitude was assessed using the Spearman's correlation index. Phylogenetic relationships among haplotypes were inferred with Network 4.5 (Fluxus Technology Ltd. at http://www.fluxus-engineering.com/sharenet.htm) by the median joining (MJ) method (Bandelt *et al.* 1999).

Genetic differentiation among populations was estimated by analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) with Arlequin software (version 3.11; Excoffier *et al.* 2005). The significance of the values was computed by a permutation test with 10,000 permutated matrices. The AMOVA was based on distances between cpSSR haplotypes, calculated as the sum of the squared number of repeat differences between two haplotypes: $d_{xy} = \Sigma[a_{xi} - a_{yi}]^2$ (where a_{xi} and a_{yi} are the number of repeats for the ith locus in haplotypes x and y). This gives Φ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin 1995) for population differentiation (Michalakis and Excoffier 1996).

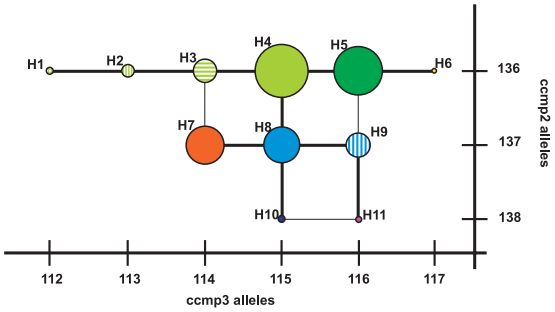

Figure 2.1: Median Joining (MJ) network for *Cistus ladanifer* cpSSR haplotypes. Axes indicate the size of ccmp2 and ccmp3 variants for each haplotype. Thin lines in each of the three loops indicate links that could be removed following the coalescence theory predictions (Crandall and Templeton 1993). Haplotypes are indicated with the same patterns as in Fig.2.2

The possible presence of geographic structure was evaluated with several tests. First, we tested for the presence of a phylogeographic structure by comparing differentiation of unordered alleles (G_{ST}) and ordered alleles (R_{ST}) with the Permut CpSSR 2.0 software (Pons and Petit 1996; http:// www.pierroton.inra.fr/genetics/labo/Software). One thousand random permutations of haplotype identities were carried out, while maintaining haplotype frequencies and the matrix of pair-wise haplotype differences as in the original study (Burban *et al.* 1999). If R_{ST}, which takes into account genetic differences between the haplotypes, is significantly higher than G_{ST}, this indicates the presence of a phylogeographical structure (Pons and Petit 1996), i.e., closely related haplotypes are more often found in the same geographical area than would be expected by chance. Second, we tested for a pattern of isolation by distance. A Mantel test with 10,000 ran-

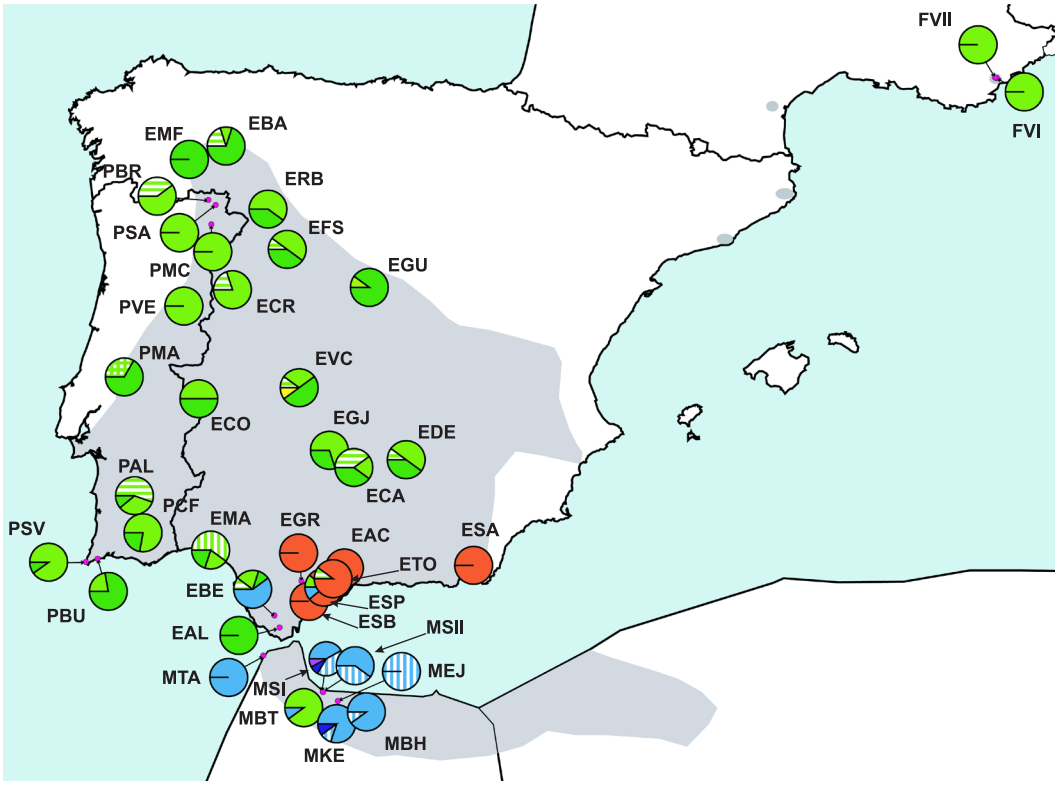

Figure 2.2: *Cistus ladanifer* cpSSR haplotype map. Grey-shaded area indicates the present natural distribution of *C. ladanifer*. The same patterns are used for haplotypes as in Fig. 2.1. In order to obtain a proper view of haplotype distribution, some radial graphs were displaced from their original position (arrows connect them with their original position). Population codes (see Table 2.1) are presented next to each radial graph.

dom permutations was performed with the matrix of pair-wise genetic differentiation between populations, $(\Phi_{ST}/(1 - \Phi_{ST}))$, and a matrix of the ln (geographic distance) with the Arlequin 3.11 software (Excoffier *et al.* 2005). Third, we used a simulated annealing procedure implemented in the spatial analysis of molecular variance (SAMOVA) algorithm (Dupanloup *et al.* 2002) to define groups of populations that are geographically homogeneous and maximally differentiated from each other. The program repeatedly seeks the composition of a user-defined number, K, of groups of geographically adjacent populations that maximises Φ_{CT}, the proportion of total genetic variance due to differences among groups of populations. The program was run for 10,000 iterations for K values from K = 2 to K = 15 from each of 100 random initial conditions. Within each of the groups defined by SAMOVA, separate AMOVA analyses were performed to partition the genetic diversity at intra- and inter-population levels. Fourth, we tested for the presence of genetic barriers among

populations using the Monmonier algorithm implemented in the BARRIER 2.2 software (Manni *et al.* 2004). Virtual points were added to the original tessellation/triangulation in order to indicate the presence of the Mediterranean Sea barrier and to enable connections to be established among populations from the South of France and central Iberian Peninsula. The distances used were pair-wise Φ_{ST} (Michalakis and Excoffier 1996). Statistical confidence for the predicted barriers

Table 2.2: Analysis of molecular variance (AMOVA) of *Cistus ladanifer* (a) considering the whole data set, (b) SAMOVA groups (K = 4), (c to e) separate analyses for three groups defined by SAMOVA analysis and (f) hierarchical geographic AMOVA comparing groups of populations to the north and south of the Strait of Gibraltar.

Source of variation	d.f.	SS	Variance components	% of total variance	P
(a) Whole data set (Φ_{ST} = 0.66)					
Among populations	37	212.70	0.57	66.35	< 0.0001
Within populations	327	94.31	0.29	33.65	
Total	364	307.01	0.86		
(b) SAMOVA results (Φ_{CT} = 0.67)					
Among SAMOVA groups	3	164.19	0.82	66.83	< 0.0001
Among pops. within SAMOVA groups	34	48.51	0.12	9.69	< 0.0001
Within populations	327	94.31	0.29	23.48	< 0.0001
Total	364	307.01	1.23		
(c) Rif group (Φ_{ST} = 0.42)					
Among populations	5	7.28	0.12	41.59	< 0.0001
Within populations	56	9.77	0.17	58.41	
Total	61	17.05	0.30		
(d) Betic group (Φ_{ST} = 0.06)					
Among populations	5	0.52	0.00	6.18	N.S.
Within populations	53	3.34	0.06	93.82	
Total	58	3.86	0.07		
(e) Western group (Φ_{ST} = 0.32)					
Among populations	24	40.71	0.15	32.18	< 0.0001
Within populations	209	65.20	0.31	67.82	
Total	233	105.91	0.46		
(f) Geographic AMOVA (Φ_{CT} = 0.25)					
Iberian Peninsula vs. N Morocco	1	35.12	0.26	25.07	0.003
Among populations within groups	36	177.58	0.48	46.97	< 0.0001
Within populations	327	94.31	0.29	27.96	< 0.0001
Total	364	307.014	1.03		

d.f. = degrees of freedom, *SS* = sum of squared deviation, *P* = level of probability of obtaining a more extreme component estimate by chance alone. *n.s.* = not significant (α = 0.05).

was obtained by resampling individuals within populations in order to obtain 100 bootstrap replicates of each genetic distance matrix. A hierarchical AMOVA was then performed in order to assess the partitioning of variance between the Iberian Peninsula and North Morocco. In addition, the genetic differences among populations were visualised through a principal coordinate analysis (PCoA) performed using GenAlEx 6.3 (Peakall and Smouse 2006) and a Euclidean genetic haploid distance similar to the genetic binary distance described by Huff *et al.* (1993).

2.3 Results

The two polymorphic microsatellites, ccmp2 (three size variants) and ccmp3 (six size variants), were combined into 11 different haplotypes among the 365 individuals analysed (Fig. 2.1). The number of haplotypes per population ranged from 1 to 4, while the mean effective number of haplotypes for the 38 populations was 1.58 (with a range of variation between 1.00 and 2.78). In addition, the average H_E and D^2_{SH} values were 0.32 (0.00–0.71) and 0.40 (0.00–5.33), respectively (Table 2.1). None of the three diversity indices used was correlated with latitude: we can find depauperated populations (with diversity values = 0.00) and also diverse populations throughout the range of *C. ladanifer*. Three haplotypes (H1, H6 and H11) were singletons, while H2 was unique to one population in southern Spain (EMA). More than 30% of the plants shared the same haplotype (H4). Two haplotypes (H4 and H8) were present on both sides of the Strait of Gibraltar, whereas haplotype H7 only appeared in populations from southeast Spain (Fig. 2.2). Median-joining analysis resulted in a complex haplotype network, with three loops (Fig. 2.1). Haplotype H8 occupies a central place within the three loops and connects the common haplotypes, H3 and H7.

Although a phylogeographic structure was not detected by the permutation analysis (G_{ST} not significantly different from R_{ST}), a strong genetic structure was observed in *C. ladanifer* populations, with high values of both G_{ST} (0.60 ± 0.06) and R_{ST} (0.57 ± 0.12). The AMOVA analysis also revealed a high and significant value for inter-population differentiation (Φ_{ST} = 0.66) (Table 2). The isolation by distance test showed that among-population differentiation increased significantly with the ln (geographical distance) (Mantel test; $P < 0.05$), although the regression accounted for only a very low proportion of the total variance ($r^2 = 0.013$). The SAMOVA analyses indicated distinct groups of genetically defined geographic areas; when K = 2, a group that comprised populations from the Betic area (EAC, EGR, ESA, ESB, ESP, ETO) was separated from the rest of the populations ($\Phi_{CT} = 0.61$). In analyses where K = 3, an additional partition was identified that subdivided the second group into two areas: one comprising populations from the South of France, the whole of the Iberian Peninsula (except the Betic area) and a population from North Morocco; the other comprising populations of *C. ladanifer* subsp. *africanus* from the Rif area ($\Phi_{CT} = 0.64$). When K = 4, a

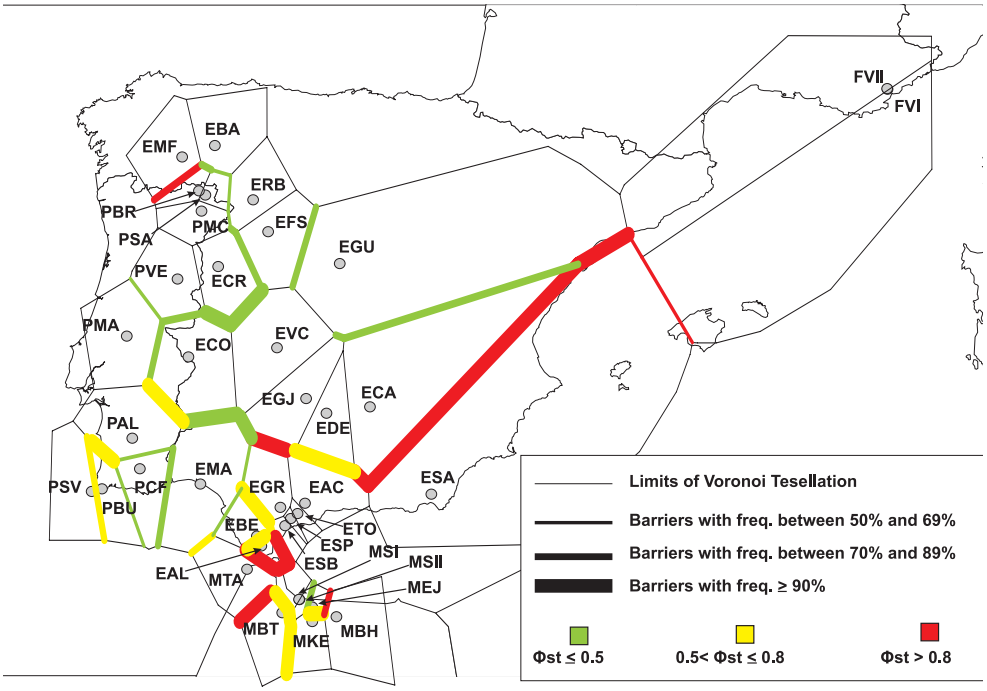

Figure 2.3: BARRIER results. Ten barriers were computed with Φ_{ST} pairwise distance matrices. The statistical support for each barrier (computed from the basis of 100 random matrices) is showed as line thickness. Only those barriers with a frequency higher than 50% were represented. The value of each barrier (Φ_{ST}) is indicated with a colour code (green, yellow, red). Populations are indicated by grey dots. The population code is indicated next to each dot (see Table 2.1). In some cases the population code is connected with an arrow to its dot.

new group comprising a single population from the southwest Iberian Peninsula (EMA) was identified (Φ_{CT} = 0.67). With value of K between 5 and 15, Φ_{CT} values did not increase significantly, and in most cases the newly defined groups comprised single populations.

Thus, SAMOVA analysis suggested the presence of three main geographic groups: one from North Morocco (hereafter Rif group), a group from the southeast Iberian Peninsula (hereafter Betic group) and a widespread group that occupies an area throughout the range of *C. ladanifer* (hereafter Western group); a fourth group is also present comprising a single population on the southwest coast of Spain (Huelva). The degree of population differentiation differed within each group (Table 2.2). Despite the short geographic distances among populations, the Rif group had the highest value of inter-population differentiation (Φ_{ST} = 0.42), whereas the widespread Western group had a lower differentiation value (Φ_{ST} = 0.32). No significant differentiation among populations within the Betic group was observed (Table 2.2). The analysis using pair-wise

Φ_{ST} distances in BARRIER software partially corroborated the SAMOVA analysis (Fig. 2.3). Although several barriers were inferred throughout the distribution area of *C. ladanifer*, the strongest genetic boundaries (with both high Φ_{ST} as well as high bootstrap support) were found in North Morocco (with barriers isolating single populations) and especially around the Betic area of the Iberian Peninsula. We stress that the BARRIER analyses separating the Iberian Peninsula and North Morocco were not inferred, although AMOVA analysis revealed that 25% of molecular variance was explained by genetic differences between populations north and south of the Strait of Gibraltar (Table 2).

Principal coordinate analysis (PCoA) extracted two axes that explained 100% of genetic variation (see Supplementary Material S 2.1). Plotting the populations along these axes revealed a grouping that matches the SAMOVA results: three groups (Betic, Rif and Western) were obtained, whereas the EMA population was plotted next to the populations from the Western cluster. This grouping has a certain taxonomic relevance. Population EBE, which belongs to *C. ladanifer* subsp. *africanus* is plotted in an intermediate position between Western and Rif group, whereas the two populations of subsp. *sulcatus* were placed together with populations of subsp. *ladanifer*.

2.4 Discussion

Chloroplast microsatellites have been widely used in phylogeographic studies (Magri *et al.* 2007, Fady *et al.* 2008, Pardo *et al.* 2008). Although some criticisms of cpSSRs arose due to problems with homoplasy, the level of homoplasy has been considered to be low enough to permit population genetic analysis (Provan *et al.* 1999a). Even when homoplasy has been identified, it was considered 'moderate' and its potential for invalidating results can be disregarded (Cuenca *et al.* 2003). Estoup *et al.* (2002), using simulations, concluded that the large amount of variability at microsatellite loci often largely compensates for homoplasious evolution, and cases in which size homoplasy may be a problem are related to high mutation rates. No estimate of mutation rates in *Cistus* is available but the lower mutation rate of chloroplast microsatellites compared to nuclear microsatellites (Provan *et al.* 1999b) suggests that size homoplasy may not be a major problem for studies at within-species level.

In this work *C. ladanifer* populations exhibited levels of genetic differentiation (G_{ST} = 0.60) that are similar to the mean and median values for maternally inherited markers in angiosperms (Petit *et al.* 2005a). Most of the molecular variance occurs between each of the four population groups, which exhibit high frequencies of haplotypes H2 (EMA), H7 (Betic group), H8 (Rif group) and H4 and H5 (Western group). From these haplotypes, it is possible that H8 is the more ancestral, since it has the largest number of connections with other haplotypes and is found in a more central position in the network (Posada and Crandall 2001). Furthermore, H8 gave rise to H7 and H4 haplotypes,

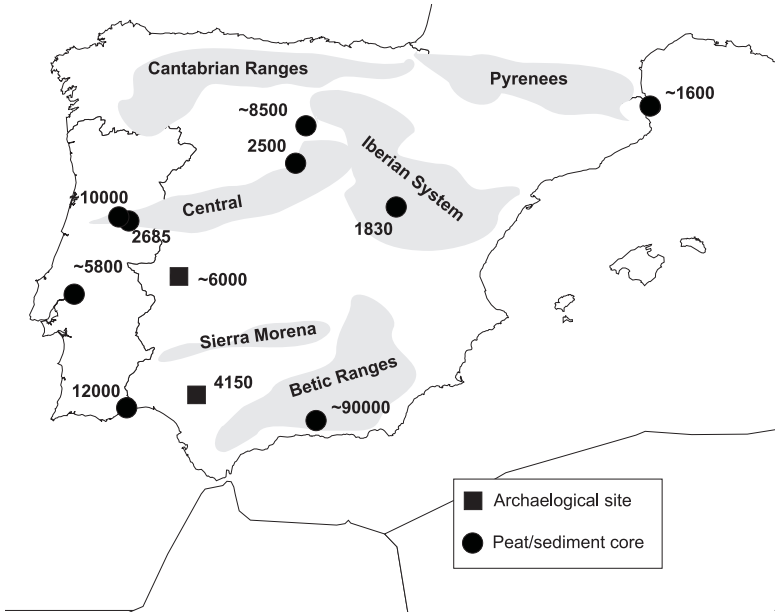

Figure 2.4: Sites and chronology of different deposits where samples of *C. ladanifer* pollen were recovered. Numbers next to each point indicate the chronology (years before present) of the first occurrence of C. ladanifer pollen in each site. The bibliographic sources of pollen data are reported in Supplementary material S 2.2.

which are the most frequent in the Betic and Western group of populations, respectively. These facts could point to the origin of the Betic and Western groups of *C. ladanifer* as a result of independent colonisation events from North Africa to the Iberian Peninsula, as proposed by Guzmán and Vargas (2009) based on chloroplast and nuclear sequence analysis.

The chronology of these colonisations is not clear, although the presence of *C. ladanifer* pollen in sites from the Betic area before the Last Glacial Maximum (around 90,000 years BP, Pons and Reille 1988, Fig. 2.4) and in southern Portugal during the Late Glacial (around 12000 years BP, Fletcher *et al.* 2007, Fig. 2.4) might indicate that it could have occurred during the Middle Pleistocene, a few million years after the opening of the Strait of Gibraltar at the end of the Messinian. Since opening (ca. 5.33 Ma, Hsü *et al.* 1977), the sea barrier of the Strait (14-km wide and about 400-m deep) has been maintained even during glacial maxima, although the lower sea level allowed emergence of different islands that reduced the width of the sea channels (Collina-Girard 2001): *C. ladanifer* should have been able to cross the Strait of Gibraltar using the emerging islands as stepping stones. The isolation effect caused by the Strait has left some traces on the genetic structure of *C. ladanifer* populations. First, three of the four groups of populations inferred by SAMOVA are present only on one side of

the Strait. Second, only two haplotypes (H3 and H8) are present both in Morocco and in the Iberian Peninsula. Finally, differentiation across the Strait accounts for 25% of the total molecular variance. However, the filtering effect of the Strait must have been lower than expected for a species like *C. ladanifer*, whose seeds tend to fall in a radius of 40 cm from the mother plant canopy (Bastida and Talavera 2002), given that the BARRIER analysis suggests that the Rif and Betic mountain ranges have acted as more effective barriers for seed flow than the Strait of Gibraltar.

The patterns of differentiation across the Strait of Gibraltar are diverse among different plant species. In a recent review, Rodríguez-Sánchez *et al.* (2008) found that establishment, rather than dispersal, may act as a key factor in genetic differentiation across the Strait. Thus, in spite of poor dispersal abilities, the high establishment potential of *C. ladanifer*, a species that can produce thousands of seeds per year that maintain viability for several years (3-year-old seeds have germination as high as 80%; personal observation, C. Quintela- Sabarís) could explain the relatively low effect of the Strait of Gibraltar on the genetic structure of its populations. In addition to the effect of the Strait, the high physiographic diversity of the northern part of Morocco (Rif Mountain ranges) and of the southern part of the Iberian Peninsula (river valleys and Betic ranges) created the geographic context for isolation during glacial periods, with the maintenance of pockets of Mediterranean taxa on south-facing slopes and in river gorges (Thompson 2005). Thus, in North Morocco (Rif region) and the south of Spain, four different clusters of populations are present. These clusters reflect the processes of post-glacial recolonisation of the Western Mediterranean area by *C. ladanifer* from its putative refugia. In the Rif region, we inferred a high degree of population differentiation and several barriers that delineate single populations (Fig. 2.3). This is congruent to an ancient presence of the species in this region and the effect of the Rif Mountains as barriers to seed flow. Regarding the Iberian Peninsula, our data suggest the occurrence of several independent glacial refugia instead of a single refugium area: first, the occurrence of three different clusters of populations (according to SAMOVA and PCoA), one of which is made up of population EMA with a unique haplotype at high frequency (0.6) on the southwest coast of Spain (this population is highly differentiated from Betic and Western clusters); second, the important genetic boundaries revealed by BARRIER analysis around the Betic region and especially in the Algeciras area (southernmost tip of the Iberian Peninsula) may indicate an area of contact between lineages expanding from different glacial refugia. In contrast with the high differences among populations in the southern Iberian Peninsula, only the Western group of populations is found in the northern part of the *C. ladanifer* natural range, suggesting that refugia in the southwest Iberian Peninsula were probably the only contributors to northward colonisation of this shrub. Populations in this area were

in a 'leading edge' position, since the lack of high mountain ranges and presence of siliceous soils in the southwest of the Iberian Peninsula favoured their expansion. The post-glacial expansion may have occurred rapidly, as illustrated by the early occurrence of *C. ladanifer* pollen in the centre of Portugal or in the Spanish Northern Meseta around 10,000–8000 BP (Van der Knaap and Van Leeuwen 1997, Franco-Mú gica *et al.* 2001, see Fig. 2.4). The presence of the related chlorotypes H4 and H5 in nearly all the populations of the Western cluster, together with the relatively low degree of population differentiation estimated among this group of populations, also support the rapid expansion model suggested by pollen data.

A stronger population genetic structure would be expected in a species with seeds dispersed mainly by barochory (Duminil *et al.* 2007), so the possible effect of endozoochory by red deer (*Cervus elaphus*; Malo and Suárez 1998) and even human activities should be taken into account as possible homogenising factors. *C. ladanifer* plays an important ecological role as coloniser in disturbed areas, so its expansion could be favoured by human-induced disturbances; thus the increase of *C. ladanifer* pollen recorded in several palaecological records is accompanied by other anthropogenic indicators, such as the increase of pollen from ruderal species (Van der Schriek *et al.* 2007) or even the decrease of *Olea* and evergreen oak pollen as a result of forest clearing by fire (Ló pez Sáez *et al.* 2007). Moreover, and related to changes in land use and microclimate variations, the presence of *C. ladanifer* pollen varied through different periods (e.g., reduction in *C. ladanifer* pollen in northeast Spain accompanied by an intense expansion of agriculture and forest cultivation from the 16th century AD; Ló pez-Sáez *et al.* 2009), a fact that could be linked to local extinctions/expansions of this plant that might have contributed to blurring of the original population genetic structure.

Moreover, *C. ladanifer* has been commercially exploited for centuries because of its fragrant resin (labdanum). The presence of *C. ladanifer* pollen in mummified remains from the 4th century AD found in Lyon (Girard and Maley 1999) as well as in cesspits from the 14th and 15th centuries in Flanders (Deforce 2006), is explained by its use as a cosmetic as well as in medicine. The intense use of *C. ladanifer* may have favoured human-mediated long-distance dispersal of this species in areas outside its natural distribution range. This may have occurred with *C. ladanifer* populations in the northeast Iberian Peninsula and southern France, where the hypothesis of artificial introduction of this species was formulated (Demoly and Montserrat 1993). Indeed, the populations analysed in southern France are fixed for the same chlorotype of populations as in the western part of the Iberian Peninsula. In contrast to expansion of the Western cluster, populations in the Betic area were encompassed by mountain ranges that acted as important barriers to the north, and may have expanded to the west, until they contacted other clusters of popula-

tions in the Algeciras area. Some authors (e.g., Petit *et al.* 2005b) proposed that colonisation of new territories might result in accelerated rates of molecular evolution and haplotype diversification, whereas the stable populations would retain ancestral characters. This could be an explanation for the lack of genetic structure, with fixation of haplotype H7 (which is directly related to the most ancestral haplotype H8) in almost all populations of this cluster. The Betic area has been identified as one of 10 hotspots of plant diversity in the Mediterranean Basin (Médail and Quézel 1997). In addition, at least two putative glacial refugia for plants have been identified in the southeast Iberian Peninsula (Médail and Diadema 2009). The latter work underlines the role of glacial refugia as climatically stable areas where we may find unique genetic diversity for plant species. Thus, for *C. ladanifer* (as for other species, such as the white oaks complex, Olalde *et al.* 2002 or *Pinus pinaster,* Bucci *et al.* 2007) the Betic area should be viewed as a 'relict' area where conservation of its populations (with a unique haplotype) should be a priority.

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Chapter 3

Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins

This chapter reproduces the published paper: Quintela-Sabarís C, Vendramin GG, Castro-Fernández D, Fraga MI (2010) Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins Plant and Soil 334:161-174

Previous page: *Cistus ladanifer* subsp. *africanus* growing in a degraded *Tetraclinis articulata* forest in the Bni Bouchra ultramafic area (Rif, N of Morocco). (Photo: C. Quintela-Sabarís)

CHAPTER 3

Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins

Keywords: Chloroplast microsatellites; *Cistus ladanifer*; genetic diversity; heavy metals; ultramafic areas

ABSTRACT

Cistus ladanifer L. (Cistaceae) is a Mediterranean shrub covering different kinds of soils in the Western Mediterranean area. This species has colonised several metalliferous areas (serpentine outcrops as well as human-polluted sites) throughout its distribution range, and is therefore an interesting species to study the possible effects on genetic diversity and differentiation produced by the colonisation of areas polluted with heavy metals. The genetic structure of 33 natural populations distributed across its entire natural distribution range (Morocco, Portugal and Spain) and growing on either metalliferous or non-metalliferous soils was investigated using chloroplast microsatellites. Population genetic parameters were estimated and genetic groups were identified using Bayesian inference. In addition, we compared the genetic diversity and differentiation among metallicolous and non-metallicolous populations within each Bayesian-defined group. The cpSSR data suggested that metallicolous populations of *Cistus ladanifer* have arisen through multiple independent evolutionary origins within two different chloroplast lineages. Evidence that the soil type provoked genetic bottlenecks in metallicolous populations or genetic differentiation among metallicolous and non-metallicolous populations was not observed. Historical factors are the main cause of the present genetic structure of *C. ladanifer*. The nature of tolerance to heavy metals as a species-wide trait in this shrub is discussed.

3.1 Introduction

Sites with high heavy metal contents in soils, either of natural origin (such as weathering of ultramafic bedrocks) or generated by anthropic activities (mining and industrial activities, atmospheric deposition, excessive use of agrochemicals or even highway traffic) (Padmavathiamma and Li 2007) are interesting areas for plant researchers due to their specific soil conditions and distinctive flora. Although some metals, such as Cu or Zn, are essential for their development, the occurrence of high contents of heavy metals has several toxic effects on plants: binding to proteins and alteration of their structure, displacement of essential elements resulting in deficiency effects or even promoting the formation of free radicals (Hall 2002). Metal toxicity, together with deficiency in nutrient contents, yield areas that are usually shallow with rocky soils and low moisture and with scarce plant cover (Brady *et al.* 2005). Thus, serpentine outcrops and mine deposits act as edaphic discontinuities in mainland regions, which have been defined as ecological or ‘edaphic islands’ (Lefèbvre and Vernet 1990).

When colonising these ‘islands’, plant populations have to cope with several environmental constraints (met-

als, dryness, isolation, etc.) that can leave their imprints on the genetic structure of plant populations. For instance, it has been proposed that plant populations in metalliferous areas can suffer a founder effect, which would significantly reduce their genetic diversity (Lefèbvre and Vernet 1990). Moreover, in some plant species, populations growing in metalliferous soils have shown significant genetic differentiation with respect to metal tolerance from neighbouring populations in 'normal' soils, even with the occurrence of substantial gene flow (Vékemans and Lefèbvre 1997, Linhart and Grant 1996 and references therein). Deng *et al.* (2007) using RAPD markers found significant genetic differentiation between mine populations and uncontaminated populations of the pseudometallophyte *Sedum alfredii*.

Plants growing in metalliferous soils can be either exclusive metallophytes (plants restricted to metalenriched habitats) or facultative metallophytes (also called pseudometallophytes), that is, species having both metallicolous (growing in metalliferous soils) and non-metallicolous populations (Wu 1990). Thus, pseudometallophytes are interesting species when studying the potential influence of environmental constraints on patterns of genetic diversity (Linhart and Grant 1996).

Several works have investigated the distribution of neutral genetic diversity within and between metallicolous populations (M) and non-metallicolous populations (NM) of different pseudometallophyte plant species (mainly herbaceous or undershrubs), using either isozymes (Wu *et al.* 1975, Ducousso *et al.* 1990, Westerbergh and Saura 1992, Bush and Barrett 1993, Vekemans and Lefèbvre 1997, Nordal *et al.* 1999, Nyberg Berglund and Westerbergh 2001) or DNA based markers (Mengoni *et al.* 2000, 2001, 2006, Pauwels *et al.* 2005).

In most cases, similar values of within-population diversity were estimated in M and NM populations, although in some works a diversity decrease was observed in M populations of *Deschampsia cespitosa* (Bush and Barrett 1993), *Lychnis alpina* (Nordal *et al.* 1999) and copper mine (but not serpentine) populations of *Silene paradoxa* (Mengoni *et al.* 2001).

Another subject that researchers have focused on, is the origin of the M populations. Analyses of diverse pseudometallophytes (Bush and Barrett 1993, Vekemans and Lefèbvre 1997, Mengoni *et al.* 2001, Pauwels *et al.* 2005) support the theory that geographically distant M populations (but even at short distances of hundreds of meters; Al-Hiyali *et al.* 1988) could have evolved independently from neighbouring NM populations; that is, M and NM populations do not constitute different phylogenetic lineages, and genetic distances among N and NM populations are a function of geographic distances between them.

These findings underline the importance of studying historical factors and population-genetic processes in order to dissect the effects of the demographic processes (such as patterns of migration or bottlenecks) from those related to selective processes (Staton *et al.* 2001).

Cistus ladanifer L. (gum rockrose) is a woody, semi-deciduous shrub growing in a wide range of latitudes, altitudes and climatic conditions in the Western Mediterranean region (South of France, Iberian Peninsula and North of Algeria and Morocco) (Demoly and Montserrat 1993). Its populations constitute early successional stages adapted to disturbances in Mediterranean ecosystems, in particular fires (Bastida and Talavera 2002). It is a pseudometallophyte that has established populations over different types of bedrock material (granites, schists, slates, etc.) and has also colonized different ultramafic areas in N Morocco (Bni Bouchra) (Ater *et al.* 2000), S Spain (Málaga) (Alados *et al.* 1999), NE Portugal (Trás-Os-Montes) (Díez Lázaro *et al.* 2006, Freitas *et al.* 2004a) and diverse mine tailings in Central to South-Western Iberian Peninsula (Murciego *et al.* 2007, Freitas *et al.* 2004b).

C. ladanifer is an entomophyllous, obligatory outcrossing species, bearing a gametophytic mechanism of incompatibility (Talavera *et al.* 1993). It is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). Three subspecies have been described based on leaf traits (Demoly and Montserrat 1993). Two subspecies, *Cistus ladanifer* subsp. *ladanifer* and subsp. *africanus*, are widespread and they have colonized M (ultramafic) areas, although only subsp. l*adanifer* is found also in mine tailings from the Iberian Peninsula. Finally *C. ladanifer* subsp. *sulcatus* (formerly *C. palhinhae*) is restricted to limestone derived soils on the coast of the southwestern tip of Portugal.

In this work, we have analysed 33 *Cistus ladanifer* populations sampled throughout the species distribution range using chloroplast (cp) DNA markers (microsatellites, SSRs). These markers are of special interest when studying colonisation patterns. Chloroplast DNA is generally maternally inherited in angiosperms, whose dispersion is therefore mediated by seeds only. In addition, the effective population size for haploid cpDNA is smaller than diploid nuclear genes, so the differentiation due to genetic drift can be stronger (Comes and Kadereit 1998) and phenomena like genetic bottlenecks can be more easily detected (Echt *et al.* 1998). For instance, cpSSR markers detected a reduction in genetic diversity within M populations of *Silene paradoxa* where RAPD markers failed (Mengoni *et al.* 2001).

These characteristics justify the wide use of cpDNA in order to infer the population history of plant populations (Petit *et al.* 2003, Magri *et al.* 2007). Once the phylogeography of the species has been inferred, a better understanding of the effect of metal pollution on the genetic structure of populations is possible, avoiding spurious correlations resulting from historical or demographic processes (Staton *et al.* 2001). This is especially interesting in *C. ladanifer*, since it colonizes M areas at different latitudes (from N Morocco to NE Portugal) in a region whose physiographic (spatial heterogeneity) and climatic diversity offers complex phylogeographic patterns (Gómez and

Lundt 2007).

In the present paper, the following main questions were addressed: Do M populations of *C. ladanifer* have the same origin? Did M populations of C*istus ladanifer* suffer a reduction in diversity? Do differences exist in the demographic effects of the colonisation of M areas along a latitudinal gradient?

3.2 Material and Methods

3.2.1 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire distribution natural range of this species were sampled. The subspecies growing in each site was identified on the basis of morphological traits. We included metallicolous (M) populations from different geographic areas: ultramafic outcrops of Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), and M populations growing on mine tailings from the centre of the Iberian Peninsula (Table 3.1). After the analyses of phyto-available trace metals, two populations (EAL and EDE), growing near highways, were included in the M group (see Fig. 3.1).

In each population, a longitudinal transect was established. Ten plants separated by at least 5 m were selected along the transect and their ripe fruits were collected. Seeds were sown and seedlings grown in a laboratory. One seedling per mother plant was selected for the subsequent analyses. Young plants were frozen in liquid nitrogen and conserved at −20°C until DNA extraction.

In addition, in each site one (or two) soil samples were collected from 5 to 15 cm in depth. Each soil sample was air-dried and sieved through a 2 mm-mesh.

3.2.2 Soil chemical analyses

Sieved soil subsamples were milled in an agatha mortar to achieve homogeneity. Total amounts of Cr, Cu, Mn, Ni, Pb and Zn in soils were quantified in solid subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF). Other subsamples were digested with HNO3 for the quantification of Co contents with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) element analysis.

In order to determine the concentration of heavy metals potentially available for plants, 10 g of dried soil was mixed with an extraction solution (Ammonium Acetate 0.5 M + EDTA 0.02 M + Acetic Acid 0.5 M, buffered at pH 4.65) (Lakanen and Erviö 1971) in a ratio of

Table 3.1: Within-population haplotypic diversity estimates. First column includes population name and code. Second column indicates *Cistus ladanifer* subspecies: *afr*: subsp. *africanus*; *lad*: subsp. *ladanifer*; *sul*: subsp. *sulcatus*. Geographic coordinates are given in decimal degrees. *M*: metallicollous population; (u): ultramafic area; (m): mine tailing; (h): highway affected area. *NM*: non-metallicollous population; *N.S.*: number of plants surveyed in each population; H_E: Nei's (1987) haplotypic diversity; $r_{(7)}$: haplotypic richness after rarefaction to the uniform sample size of 7 (El Mousadik and Petit, 1996); D^2_{SH}: average genetic distances among individuals (Vendramin *et al.* 1998); *Overall Mean*: mean for the whole set of populations ± standard deviation values.

Population (Code)	Subsp	Long	Lat	Soil Type	Substratum	N.S.	H_E	$r_{(7)}$	D^2_{SH}
Ketama (MKE)	*afr*	4.64º W	34.95º N	NM	Schists	10	0.38	1.40	0.22
Bni Hadifa (MBH)	*afr*	4.17º W	35.02º N	NM	Sandstones	10	0.2	0.70	0.1
Bab Tazaa (MBT)	*lad*	5.24º W	35.08º N	NM	Micaschists	10	0.2	0.70	0.1
El Jebha (MEJ)	*afr*	4.64º W	35.18º N	NM	Sandstone	10	0	0	0
East Bni Bouchra (MSII)	*afr*	4.89º W	35.29º N	M (u)	Serpentinised peridotite	12	0.7	2.16	0.58
West Bni Bouchra (MSI)	*afr*	4.90º W	35.30º N	M (u)	Serpentinised peridotite	10	0.53	1	0.27
Tanger (MTA)	*afr*	5.93º W	35.78º N	NM	Sandstone	10	0	0	0
Almodóvar (EAL)	*lad*	5.65º W	36.16º N	M (h)	Clays close to a road	10	0	0	0
Benalup (EBE)	*afr*	5.73º W	36.32º N	NM	Sandstone	10	0.64	2.33	0.76
Sierra Bermeja (ESB)	*lad*	5.18º W	36.48º N	M (u)	Serpentinised peridotite	10	0	0	0
Sierra Palmitera (ESP)	*lad*	5.07º W	36.60º N	M (u)	Serpentinised peridotite	9	0.42	1.56	0.5
Sierra de Tolox (ETO)	*lad*	4.93º W	36.68º N	M (u)	Serpentinised peridotite	10	0.2	0.70	0.1
Grazalema (EGR)	*lad*	5.27º W	36.78º N	NM	Decarbonated limestone	10	0	0	0
Sierra de Aguas (EAC)	*lad*	4.79º W	36.84º N	M (u)	Serpentinised peridotite	10	0	0	0
São Vicente (PSV)	*sul*	8.98º W	37.03º N	NM	Limestone	10	0.2	0.70	0.1
Burgau (PBU)	*sul*	8.78º W	37.07º N	NM	Limestone	9	0.39	0.97	0.19
Mazagón (EMA)	*lad*	6.84º W	37.15º N	NM	Sand deposits	10	0.62	1.87	1.78
Corte Figueira (PCF)	*lad*	8.03º W	37.39º N	NM	Schists	9	0.39	0.97	0.19
Aljustrel (PAL)	*lad*	8.18º W	37.88º N	M (m)	Pyrite mine tailing	9	0.64	1.78	0.53
Cardeña (ECA)	*lad*	4.36º W	38.28º N	NM	Granite	10	0.71	1.93	0.89
Despeñaperros (EDE)	*lad*	3.51º W	38.39º N	M (h)	Quartzites, close to the highway.	10	0.64	1.70	0.46
El Guijo (EGJ)	*lad*	4.77º W	38.52º N	NM	Slates	10	0.47	0.99	0.23
La Codosera (ECO)	*lad*	7.08º W	39.19º N	M (m)	Sb mine tailing	10	0.56	1	0.28
Valdecaballeros (EVC)	*lad*	5.34º W	39.33º N	NM	Sedimentary material (gravels, clays)	10	0.71	2.39	0.71
Vela (PVE)	*lad*	7.29º W	40.43º N	NM	Granite	9	0	0	0
Ciudad Rodrigo (ECR)	*lad*	6.49º W	40.63º N	NM	Quartzites	10	0.36	0.93	0.18
Guadarrama (EGU)	*lad*	4.10º W	40.68º N	NM	Granite	10	0.2	0.70	0.1
Fuente Saúco (EFS)	*lad*	5.51º W	41.19º N	NM	Sandstone and conglomerates	10	0.64	1.70	0.46
Macedo dos Cavaleiros (PMC)	*lad*	6.82º W	41.52º N	M (u)	Serpentinised peridotite	10	0	0	0
Ricobayo (ERB)	*lad*	5.81º W	41.70º N	NM	Quartzites and filites	10	0.53	1	0.27
Samil (PSA)	*lad*	6.75º W	41.78º N	M (u)	Serpentinised peridotite	7	0	0	0
Bragança (PBR)	*lad*	6.87º W	41.85º N	M (u)	Dunite	10	0.53	1	0.27
Monte Furado (EMF)	*lad*	7.20º W	42.39º N	NM	Schists	10	0	0	0
					Overall mean ± SD		0.31 ± 0.27	0.91 ± 0.77	0.27 ± 0.36

soil:extraction solution of 1:5. The suspension was shaken for 30 min, after which it was allowed to stand for at least half an hour and was then filtered through paper (Albet DP 145). The filtrate was stored cold to be analysed with an atomic absorption spectrophotometer (AAS). The following available trace metals were determined with AAS: Co, Cr, Cu Mn, Ni, Pb and Zn.

3.2.3 DNA extraction

DNA was extracted from 100 mg of frozen leaves using Dneasy® Plant Mini Kit (QIAGen), following the manufacturer's indications. In several cases an additional wash with 500 µl of absolute ethanol was needed in order to remove secondary compounds from the DNA extracts.

3.2.4 Microsatellite analysis

In an initial screening, universal cpSSR primers ccmp1 to ccmp10 (Weising and Gardner 1999) and Fagaceae cpSSR primers cmcs 1 to 14 (Sebastiani *et al.* 2004) were tested on a subset of 30 samples from 6 geographically distant populations. Only primers ccmp1, ccmp2, ccmp3, ccmp5, ccmp10 and cmcs1 yielded consistent amplifications, of which ccmp1, ccmp5, ccmp10 and cmcs1 were monomorphic. The two polymorphic cpSSRs were then used to amplify all samples of the 33 populations. Amplification reactions were performed in 12.5 µl total volume using 10 ng of template DNA, 1× reaction buffer (Promega, Madison, WI, USA) containing 1.5 mM of MgCl2, 0.2 µM of each primer, 0.2 mM of each dNTP, 1% of bovine serum albumin, and 0.5 U of GoTaq® DNA Polymerase (Promega). DNA was amplified with the following thermal profile: one denaturation cycle of 4 min at 95°C, followed by 25 cycles each consisting of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s with a final extension step at 72°C for 8 min. PCR products were loaded on a capillary automatic sequencer MegaBACE 1000 (GE Healthcare Biosciences). MegaBACE ET400 (GE Healthcare Biosciences) was used as size standard. Fragment lengths were determined using the MegaBACE FRAGMENT PROFILER software version 1.2 (GE Healthcare Biosciences).

3.2.5 Data analysis

A Principal Component Analysis (PCA) was performed in order to aggregate populations according to (1) total contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn in soils (referred here as CoT, CrT, CuT, MnT, NiT, PbT and ZnT) or (2) Ammonium Acetate/EDTA metal extractable contents (referred here as CoE, CrE, CuE, MnE, NiE, PbE and ZnE). Values below detection limits were recorded as 0.1 $\mu g.g^{-1}$ for statistical analysis. With the PCA, we reduced the dimensionality of the data, retaining, in our case, two first Principal Components (PCs) that contribute most to the variance of soils. A Varimax rotation was applied to the PCAs in order to simplify the interpretation of the extracted Principal Components.

Phylogenetic relationships among haplotypes were inferred with NETWORK 4.5 (Fluxus Technology Ltd. at www.fluxus-engineering.com) using the median

joining (MJ) method (Bandelt *et al.* 1999). We applied the three criteria (frequency, topology and geography) proposed by Pfenninger and Posada (2002) in order to remove loops or ambiguities in the haplotype network.

Different parameters of genetic diversity within populations were estimated: (1) the haplotypic diversity (H_E = $[n/(n-1)][1-\Sigma p_i^2]$, (where n is the number of individuals analysed in a population and p_i is the frequency of the i-th haplotype in a population; Nei 1987), (2) the haplotypic richness $r_{(n)}$, which is obtained after rarefaction to a uniform sample size of n (in our study, the value of n was fixed at 7, the lowest size of the analysed populations), as described in El Mousadik and Petit (1996), and (3) the D^2_{SH} measure, as defined by Vendramin *et al.* (1998), which takes into account the difference in the number of repeats among the different cpDNA haplotypes considered.

In addition, the distribution of the pairwise cpSSR repeat length differences among individual plants, totalled over all two cpSSR loci within an individual plant, was plotted to compare different patterns. Genetic differentiation among populations was estimated by the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) using Arlequin software (version 3.11; Excoffier *et al.* 2005). The significance of the values was computed by a permutation test from 10,000 permutated matrices. The AMOVA was based on distances between cpSSR haplotypes, calculated as the sum of the squared number of repeat differences between two haplotypes: $d_{xy} = \Sigma[a_{xi} - a_{yi}]^2$ (where a_{xi} and a_{yi} are the number of repeats for the ith locus in haplotypes x and y). This gives Φ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin 1995) for population differentiation (Michalakis and Excoffier 1996).

The Cavalli-Sforza and Edwards distances based on haplotype frequencies were used to construct a Neighbour Joining (NJ, Saitou and Nei 1987) dendrogram with the Populations software (O. Langella, UMR de Génétique Végétale, Ferme du Moulon, Gif/Yvette, France). To test for node robustness, bootstrapping was performed on individuals using 1,000 resamplings.

In order to infer population genetic structure, Bayesian analysis using a spatial clustering model implemented in BAPS software version 5.2 was performed (Corander *et al.* 2008). These authors have shown that the spatial model improves the statistical power to detect the underlying population structure when dealing with a low number of loci.

The spatial clustering of groups model was run using each population, with known coordinates, as the unit to be clustered. We initially fixed k (the number of clusters) from 2 to 25. We then selected the value of k that had the minimum log marginal likelihood and re-ran the analysis 100 times to obtain the optimal partition of populations. A neighbour-joining tree was then constructed (Saitou and Nei 1987) with the Kullback–Leibler divergence matrix provided as output with BAPS. This matrix can be used as a measure of relative genetic distance between the BAPS-identi-

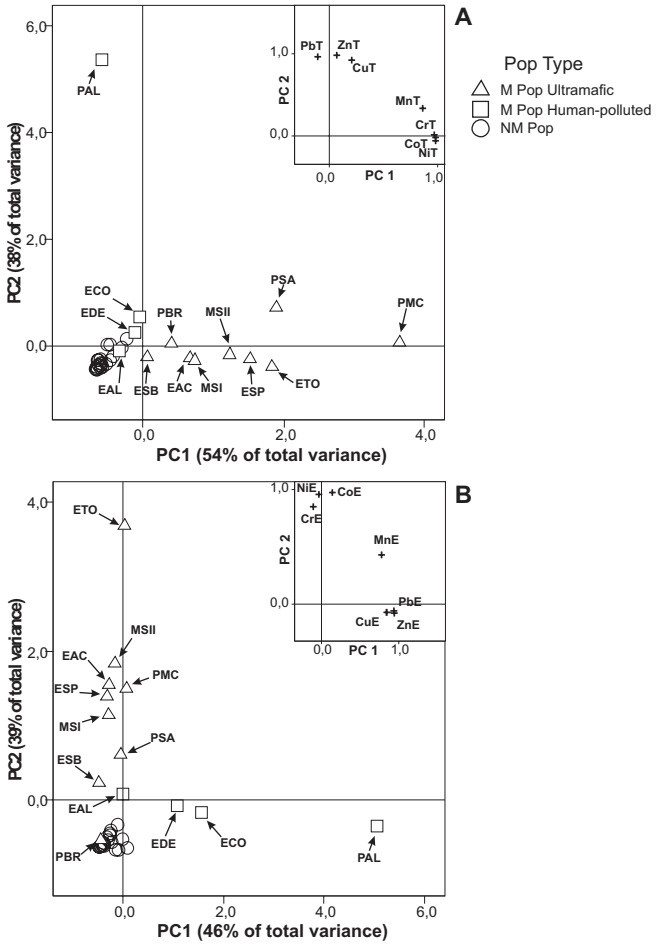

Figure 3.1: Principal Component Analysis (PCA) results. Populations are ordinated according to the total (Fig. 1a) or Ammonium Acetate/EDTA extractable (Fig. 1b) quantities of heavy metals in their soils. The percentage of variance explained by each axis is reported. Circles indicate non-metallicolous (NM) populations, whereas triangles and squares indicate metallicolous (M) populations from ultramafic areas and human-polluted soils, respectively. The codes of M populations are also indicated. In the upper right-hand corner of each graph the loading of each metal on each of the PCs is reported.

fied clusters (BAPS 5.2 manual distributed with the program).

Taking into account the grouping performed by BAPS, we (1) compared the levels of intra-population diversity of M and NM populations using an analysis of variance (ANOVA) and (2) tested the differentiation of M and NM populations with additional separate AMOVA analyses (Excoffier *et al.* 1992). With this two step-approach (BAPS and ANOVA/AMOVA) we tried to extract first the effect of phylogeography and then analyse the effect of colonisation of metallicolous areas within phylogeographic homogeneous groups of populations, thus avoiding confounding effects of phylogeography on the effect of metal pollution over population genetic structure.

Isolation-by-distance patterns between populations were tested considering all populations and then considering M and NM populations separately. A Mantel test with 10,000 random permutations was performed with the matrix of pairwise genetic differentiation between populations,

using $\Phi_{ST}/(1 - \Phi_{ST})$, and a matrix of the logarithmically (ln) transformed geographic distance. AMOVA and Mantel tests were computed with the Arlequin software version 3.11 (Excoffier *et al.* 2005).

3.3 Results

3.3.1 Soil characteristics

The results of the analyses of total and Ammonium Acetate/EDTA extractable metals in soils for each population are presented in the Supplementary Material S 3.1. On the basis of these data, we conducted two Principal Component Analyses (PCA).

The PCA performed on total metal contents (Fig. 3.1a) extracted two principal components (PCs) that explained 92% of total variance. The first Principal Component (PC1; 54% of total variance) was mainly composed of the total contents of Co, Cr, Ni, and Mn, suggesting that this axis is related to the ultramafic nature of soils. The second PC (PC2; 38% of total variance) was related to the degree of pollution due to human activities, since the metals with higher loadings were Cu, Pb and Zn.

The PCA based on extractable metals showed a similar pattern (Fig. 3.1b). Two PCs explaining 85% of total variance were extracted. In this case, the first PC (PC1; 46% of total variance) was related to human pollution (extractable Cu, Pb and Zn were the main contributors to this PC) whereas the second PC (PC2; 39% of total variance) reflected the ultramafic origin of soils, since the extractable contents of Co, Cr and Ni contributed significantly

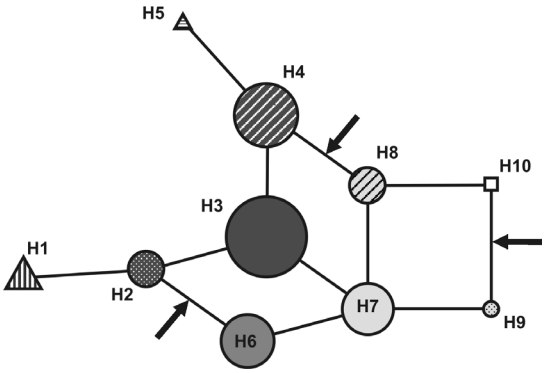

Figure 3.2: Median Joining (MJ) network of *Cistus ladanifer* cpSSR haplotypes. Haplotypes are identified with the same colours as in Fig.3.3. *Circles*: haplotypes present both in M and NM populations. *Triangles*: haplotypes exclusive to NM populations. *Square*: haplotype present only in M population. Symbols' sizes are proportional to the absolute haplotype frequency in the whole sample. Arrows indicate the connections between haplotypes that can be removed following the criteria of Pfenninger and Posada (2002).

in explaining the observed variance. The manganese content showed similar loadings on both axes.

According to the defined Principal Components, in both cases (total or extractable metal contents) NM populations were plotted in a dense swarm placed mainly in the negative values on both axes, whereas the M populations showed high scores along one of the PCs, depending on their nature (ultramafic area or human-polluted site) (Fig 3.1a and b).

On the other hand, three populations (EAL, EDE and PBR) showed differences between the two PCAs. Population PBR, whose bedrock material are dunites (a type of peridotite) is clearly separated from the NM populations based on total metal contents. Thus, it is plotted along

the axis of ultramafic populations in Fig 3.1a. In contrast, in Fig 3.1b (PCA based on extractable metal contents) this population is plotted with the NM populations. This population was treated as M because of the high total contents of metals Cr, Mn and Ni (944, 1,578 and 1,151 $\mu g.g^{-1}$, respectively) in the analysed soil samples.

The two other populations (EAL and EDE) were initially considered as NM (taking into account bedrock material and total metal contents, see Supplementary Material S 3.1 and Fig. 3.1a). Nevertheless, when performing the PCA on Ammonium Acetate/EDTA extractable metal contents, these two populations were separated from the group of NM populations (Fig 3.1b). These populations, affected by highway traffic, were consequently classified as M. EDE has an extractable Pb of more than 100 $\mu g.g^{-1}$, whereas EAL possesses in its soil moderate extractable contents of all the analysed metals.

3.3.2 Genetic diversity and structure

The two polymorphic microsatellites ccmp2 and ccmp3 yielded 3 and 5 size variants, respectively.
According to the nature of these microsatellites (both are mononucleotide repeats; Weising and Gardner 1999) the sizes of alleles varied by one base-pair.

The variants found in each microsatellite locus combined into 10 different haplotypes (see Supplementary Material S 3.2 for the definition of each haplotype). They are connected through a complex haplotype network, with 3 loops and no missing haplotypes (Fig. 3.2).

Only two haplotypes (H3 and H7) are present both in the Iberian Peninsula and North of Morocco. Haplotypes H2 to H4 are distributed across the Iberian Peninsula, whereas haplotypes H8 to H10 are found in North of Morocco and H6 is restricted to south-eastern Spain (Betic Area) (Fig. 3.3). Three haplotypes (H1, H5 and H10), found on the tips of the network, were exclusive to one population type (M or NM) (Fig. 3.2). Two of these haplotypes (H5 and H10) were singletons, whereas H1 is exclusive to NM population EMA from south-western Spain.

The geographic distribution and the frequency of haplotypes, together with a criterion of topology (Pfenninger and Posada 2002) were employed to remove one of the edges in each of the 3 loops inferred in the haplotype network and thus resolve the uncertainties in the network (Fig. 3.2).

Bayesian analysis yielded an ideal grouping with 8 clusters (Fig. 3.4), of which clusters 1, 3, 4, 6, and 7 included both M and NM populations. The NJ tree grouped these 8 clusters again in two diverging lineages of populations. The first lineage (hereafter referred to as 'South lineage') comprises populations in which H6 to H10 haplotypes are dominant (clusters 7, 8, 6 and 2), whereas the other lineage (hereafter referred to as 'North lineage') includes those populations with a high frequency of H1 to H5 haplotypes (clusters 4, 1, 3 and 5). These lineages have a taxonomic support, since the 'South lineage' comprises populations of *C. ladanifer* subsp *africanus*, together with populations

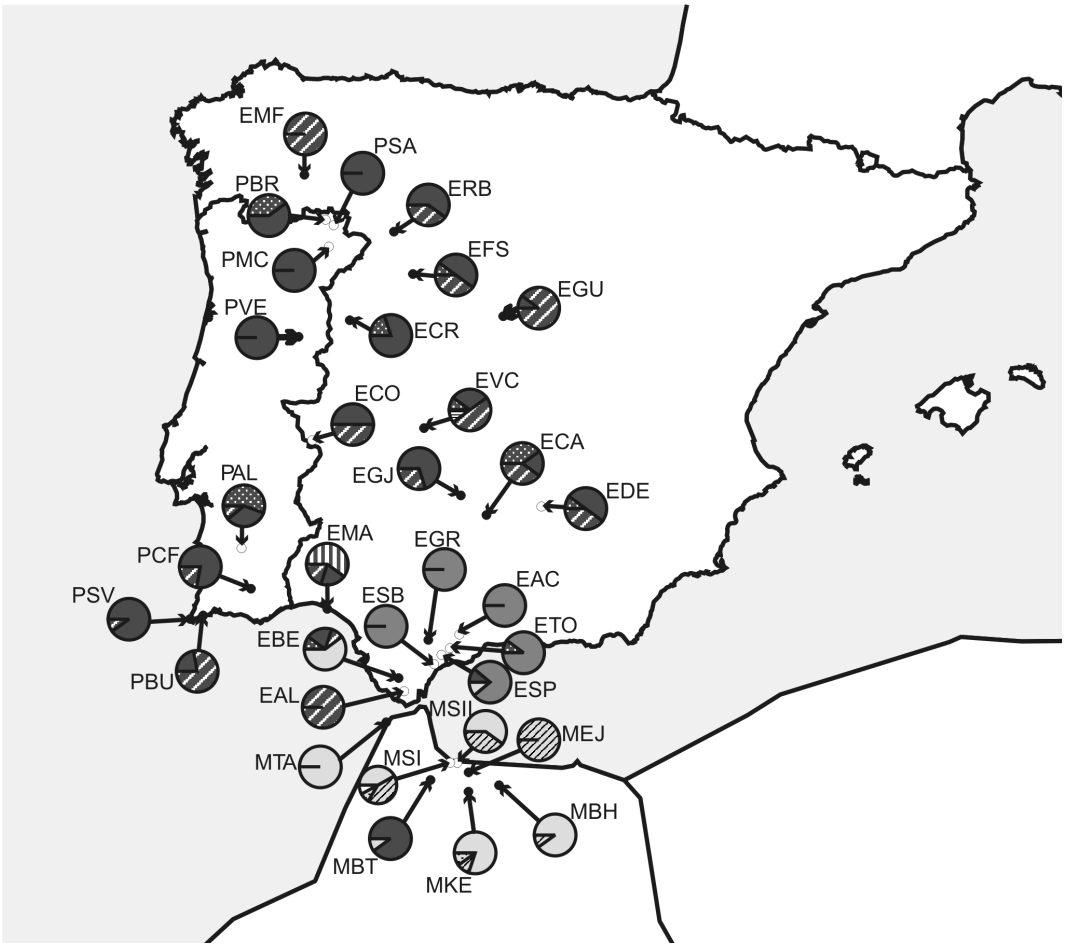

Figure 3.3: Geographic distribution of *Cistus ladanifer* cpSSR haplotypes. Colour patterns used are the same as in Fig 3.2. In order to obtain a proper view of haplotype distribution, radial graphs were displaced from their original position (arrows connect them with their original position). Next to each graph the population code is indicated (see Table 3.1). Metallicolous (M) populations are noted as white dots, non-metallicolous (NM) ones as black dots.

of *C. ladanifer* subsp. *ladanifer* growing in the Betic area, whereas the 'North lineage' is composed of populations of *C. ladanifer* subsp. *sulcatus* and *C. ladanifer* subsp *ladanifer* from the Iberian Peninsula, plus one population of subsp. *ladanifer* from the N of Morocco.

The AMOVA analysis performed on the whole sample of populations indicate that most of the molecular variation is found among populations (Φ_{ST}=0.69, Table 3.2a), that is a value similar to the mean value for angiosperms (Petit *et al.* 2005). Differentiation between edaphic types were not significant either when the whole set of populations (33 pops) was considered (Table 3.2b), nor when separate AMOVA analyses within each of the

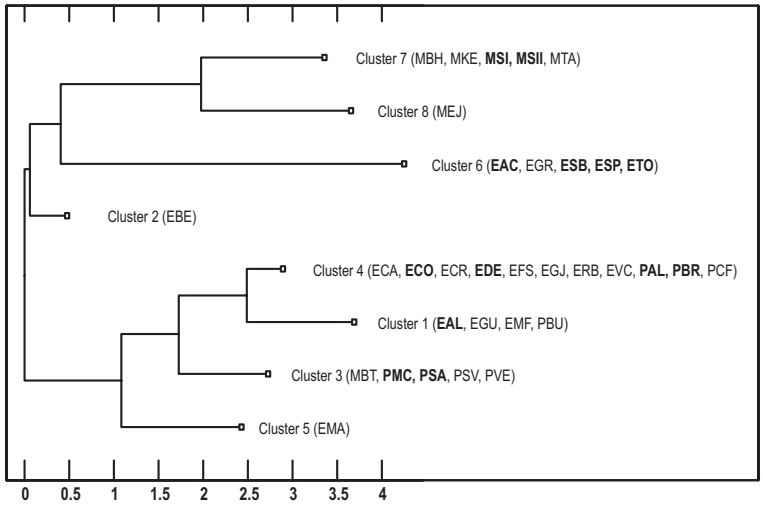

Figure 3.4: BAPS-based clustering and relationships among clusters based on the Kullback–Leibler divergence matrix. Clusters of populations were constructed with a spatial clustering model (Corander *et al.* 2008). Codes in brackets indicate populations included within each cluster. Population codes are the same as in Table 3.1. M populations are indicated in **bold type**. Horizontal scale bar indicates Kullback-Leibler distances among clusters.

lineages (North and South, Table 3.2c and d) were performed.

The NJ tree further confirmed that M and NM populations did not constitute distinct genetic groups (Fig. 3.5). M populations appear dispersed among NM populations, forming clusters that are partially congruent with BAPS results. In most cases, M populations are clustered with geographically close NM populations and were genetically distant to M populations from other geographic areas; on the other hand populations PMC, PSA and PVE (from the NE and C of Portugal) were clustered with population MBT from the N of Morocco.

No isolation-by-distance pattern was detected by Mantel's tests, irrespectively of whether we considered M populations ($P = 0.12$), NM populations ($P = 0.23$) or the whole set of populations ($P = 0.23$).

The number of haplotypes per population ranged from 1 to 4. The different estimators of within population diversity (H_E, $r_{(7)}$ and D^2_{SH}) displayed overall mean values of 0.31±0.27, 0.91±0.77 and 0.27±0.36, respectively (Table 3.1). The Analysis of Variance (ANOVA) showed no significant differences of diversity among M and NM populations considering either the whole set of populations or the lineages defined by BAPS (North, South) separately (Table 3.3). In addition, there were no qualitative differences among mismatch distributions from M and NM populations (see Supplementary Material S 3.3).

3.4 Discussion

Over the last 50 years pseudometallophytes have been studied as models of microevolution and of regional differentiation among plants. The main aim of these works has been, on the one hand, to test whether the colonisation of areas with heavy metals may result in genetically depauperated populations (as a result of

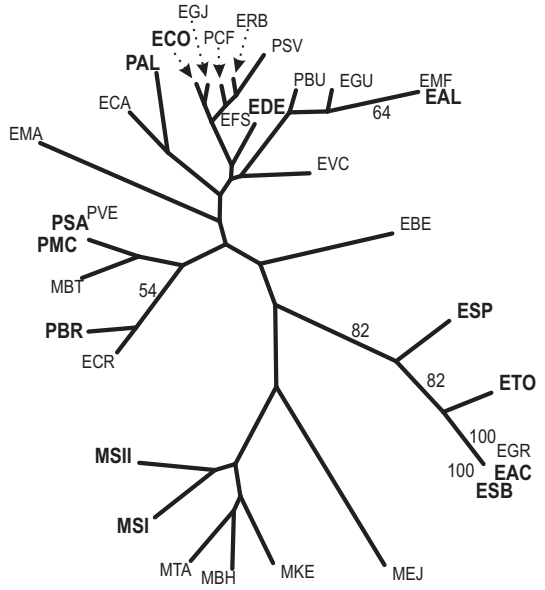

Figure 3.5: Neighbour-Joining consensus tree of populations. Cavalli-Sforza and Edwards distances between populations were used. Only bootstrap values higher than 50 are reported. M populations are indicated in **bold type**.

bottlenecks and genetic drift), and on the other, to determine whether the metalliferous populations of a particular species arose from a single micro-evolutionary event, or whether they resulted from processes of regional differentiation. Our study on the Mediterranean shrub *Cistus ladanifer*, which encompasses almost all of its natural distribution, provides evidence for the colonisation routes of metalliferous areas by this species.

Using Bayesian methods, we infer 2 population lineages with different haplotype compositions showing partial taxonomic significance ('South' lineage made up of the *C. ladanifer* subsp. *africanus* and subsp. *ladanifer* from the Betic area; 'North' lineage, made up of populations of *C. ladanifer* subsp. *ladanifer* and subsp. *sulcatus*).

The three *C. ladanifer* subspecies have been classified on the basis of leaf traits (leaf shape, nerve type and length of petiole, Demoly and Montserrat 1993). Our results showed an incongruence between molecular information and morphological traits: in fact populations of subspecies ladanifer from Betic area are placed within the cluster of subspecies africanus. This incongruence not only refers to chloroplast markers, but also to AFLP markers analysed on the same 33 populations included in this work (manuscript in preparation), thus suggesting that additional studies are needed to determine the taxonomic status for these subspecies.

In addition to the taxonomic considerations, several evidences indicate that the edaphic type does not influence the occurrence of the lineages: haplotypes specific to one single type of soil only appear on the tips of the haplotype network. Moreover, within the 'North' and 'South' lineages, as well as in the clusters defined by NJ dendrogram, both M and NM populations are found.

The two lineages detected in this study are congruent to those found by Guzmán and Vargas (2009) analysing the sequences of several chloroplast regions of *Cistus ladanifer*: these authors inferred two main chloroplast lineages, referred to as 'European' and 'African', and found that populations from the Betic area display an exclusive haplotype which belong to the 'African' lineage. Likewise, the two lineages we detected have the same

Table 3.2: Analysis of molecular variance (AMOVA) of *Cistus ladanifer*. We considered (a) all the populations (Φ_{ST}), (b) hierarchical analysis of variance among populations within edaphic types (Φ_{SC}) and between edaphic types (Φ_{CT}) and (c) (d) separate hierarchical analyses considering the clustering obtained by BAPS. *d.f.* = degrees of freedom, *SS* = sum of squared deviation, *Sig.* = probability of obtaining a more extreme component estimate by chance alone. *n.s.*, not-significant (*α value*=0.05)

Source of variation	d.f.	SS	Variance components	% Total Variance	Φ statistics	Sig.
(a) Whole data set						
Among populations	32	191.15	0.58	68.69	Φ_{ST} = 0.69	<0.0001
Within populations	291	77.14	0.26	31.31		
Total	323	268.29	0.85			
(b) M vs NM						
Among groups	1	10.16	0.03	3.23	Φ_{CT} = 0.03	n.s.
Among pops within groups	31	180.99	0.57	65.97	Φ_{SC} = 0.68	<0.0001
Within populations	291	77.14	0.26	30.80	Φ_{ST} = 0.69	<0.0001
Total	323	268.29	0.86			
(c) South cluster						
Between groups (M vs NM)	1	6.93	0.03	4.59	Φ_{CT} = 0.05	n.s.
Among pops within groups	10	50.32	0.48	71.62	Φ_{SC} = 0.75	<0.0001
Within populations	109	17.51	0.16	23.80		<0.0001
Total	120	74.76	0.67			
Among pops irrespective of groups					Φ_{ST} = 0.76	<0.0001
(d) North cluster						
Between groups (M vs NM)	1	0.36	-0.02	-4.63	Φ_{CT} = -0.04	n.s.
Among pops within groups	19	48.89	0.23	43.41	Φ_{SC} = 0.41	<0.0001
Within populations	182	59.63	0.33	61.22		
Total	202	108.88	0.53			
Among pops irrespective of groups					Φ_{ST} = 0.40	<0.0001

geographical distribution as reported by Guzmán and Vargas (2009); moreover haplotype H6, which is exclusive to populations from the Betic area, is connected to haplotype H7, common in the North of Morocco.

Thus, the 'North' and 'South' lineages seem to be more related to the existence of diverse glacial refugia for *Cistus ladanifer* located in the N of Morocco and in the Southeast (Betic area) and Southwest of the Iberian Peninsula, areas indicated as refugia for several other Mediterranean taxa (Médail and Diadema 2009).

Therefore, the extant M populations of *Cistus ladanifer* have arisen through distinct foundation events in different geographical regions and within different postglacial recolonisation lineages.

The independent origin of the M

populations of a given species seems to be common in pseudometallophytes (Mengoni *et al.* 2001, Nyberg Berglund and Westerbergh 2001, Vekemans and Lefèbvre 1997), even at a scale of hundreds of metres (Al-Hiyali *et al.* 1988). Interestingly, we found M populations within two chlorotype lineages while, for example, in *Arabidopsis halleri*, a model pseudometallophyte, all M populations, of independent origins, come from one chlorotype lineage only (north of the Alps) (Pauwels *et al.* 2005, 2008).

Most of the works on genetic structure of pseudometallophytes did not reveal evidence of genetic bottlenecks in M populations (for review, see Vekemans and Lefèbvre 1997, Mengoni *et al.* 2000). These results were mainly based on nuclear markers (isozymes or RAPDs), so the possible founder effect at nuclear loci may be eroded by subsequent pollen flow from neighbouring NM populations which could increase genetic variation in M populations. In contrast, chloroplast markers are (generally) maternally inherited, so they will only reflect the effect of seed flow. To our knowledge, only two other studies have analysed pseudometallophytes using chloroplast markers, obtaining contrasting results: Mengoni *et al.* (2001) detected a founder effect in *Silene paradoxa* populations growing on copper mine deposits, whereas Pauwels *et al.* (2005) did not find any difference in genetic diversity between M and NM populations of *Arabidopsis halleri*.

To explain these different patterns of diversity between *S. paradoxa* and *A. halleri*, Pauwels *et al.* (2005) proposed that the colonisation of metalpolluted environments is associated with a genetic bottleneck in species with populational tolerance (that is, species in which metal tolerance is found only in those populations growing on metalliferous soils), whereas in species with constitutive (or "specieswide") tolerance (such as *A. halleri*) the effect of a bottleneck may not be detected.

In the case of *Cistus ladanifer*, once we had extracted the phylogeograph-

Table 3.3: ANOVA results. The table presents the mean values for M (Metallicolous) and NM (Non-Metallicolous) of within-population diversity indexes for each cluster of populations (defined after Bayesian analysis), and for the whole set of populations. H_E: Nei's (1987) haplotypic diversity. $r_{(7)}$: allelic richness (El Mousadik and Petit, 1996) with a fixed rarefaction size of 7. D^2_{SH}: average genetic distances among individuals (Vendramin *et al.* 1998). *Sig.*: significance value. *n.s.*: not-significant (*α value*= 0.05).

		H_E			$r_{(7)}$			D^2_{SH}		
		M	**NM**	**Sig.**	**M**	**NM**	**Sig.**	**M**	**NM**	**Sig.**
Cluster defined by BAPS	South	0.308	0.203	n.s.	0.903	0.738	n.s.	0.242	0.180	n.s.
	North	0.339	0.387	n.s.	0.783	1.061	n.s.	0.220	0.371	n.s.
Whole data		0.325	0.332	n.s.	0.838	0.964	n.s.	0.230	0.314	n.s.

ic effect through the Bayesian analysis, we did not detected any significant differentiation nor significant differences in the genetic diversity between edaphic types, even in the M populations of more recent origin, i.e. populations growing on mine tailings from the Iberian Peninsula.

These inferences may suggest that M populations were founded recently by a high number of individuals, or that the foundation events are antique but in the presence of a significant seed flow (cpDNA is maternally inherited in C*istus ladanifer*; Guzmán and Vargas 2009) from neighbouring NM populations that masked the effect of genetic bottlenecks and limited genetic differentiation between edaphic types.

Both hypotheses assume that the metalliferous areas do not exert selective pressures on *Cistus ladanifer*; however, it should be stressed that variation of neutral markers, like cpSSRs, cannot be related to variation of adaptive traits such as tolerance to heavy metals (Le Corre and Kremer 2003), as it occurred in *Thlaspi caerulescens* (Jiménez-Ambriz *et al.* 2007). Thus, genetic differentiation between M and NM populations at genes related to metal-tolerance can be significantly underestimated.

Nevertheless, as discussed by Vekemans and Lefèbvre (1997), after a bottleneck the number of alleles at neutral loci increases as a result of new mutations, when population size increases. This phenomenon can be observed in the pseudometallophyte *Silene paradoxa* (Mengoni *et al.* 2001): 8 populations (5 serpentine outcrops, 2 copper mines, 1 nonmetallicolous) separated up to 205 km showed 13 cpSSR haplotypes exclusive to one population (of 27 haplotypes detected) and no haplotypes were shared by all populations. In contrast, in the case of *C. ladanifer* only a singleton was exclusive of M populations. This fact suggests that the inferred levels of diversity cannot be caused by mutations after a bottleneck following the colonisation of metalliferous areas.

To conclude, following Pauwels *et al.* (2005) and considering the multiple origins of M populations and the lack of bottlenecks, we propose that the tolerance to heavy metals could be a characteristic of *Cistus ladanifer*, even though this plant is more commonly found in non-metalliferous areas, as already observed in *Thlaspi montanum* (Boyd and Martens 1998) or in *Andropogon virginicus* (Gibson and Risser 1982). Indeed, our hypothesis is supported by the results obtained by Kidd *et al.* (2004), who observed that nonmetallicolous populations from North of Portugal showed high tolerance to Cu and Zn in hydroponic cultivation.

Taking into account our findings on *Cistus ladanifer* and those obtained by other authors, we believe that this plant could be very useful in the recovering of degraded soils in the Mediterranean region, also considering that it has other interesting traits (Frérot *et al.* 2006). First, *C. ladanifer* is adapted to water stress (Nuñez-Olivera *et al.* 1996) so it can cope with a long, dry summer season. Second, it has a high growth rate and productivity (Patón *et al.* 1998) when conditions

permit, developing dense root and shoot systems that can limit soil erosion. And third, it is a native species of Western Mediterranean flora; therefore, its use in phytoremediation would not imply any potential threats to ecosystems deriving from the use of alien species (Méndez and Maier 2008; and references therein), an important issue in the biodiversity-rich Mediterranean area.

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Chapter 4

Heavy metal accumulation in leaves of divergent chloroplast lineages of the pseudometallophyte *Cistus ladanifer* L. Implications for phytostabilization

Previous page: General view of an old pyrite mine tailing in Aljustrel (Alentejo, S Portugal). In this tailing we quantified (total contents): 752 $mg \cdot kg^{-1}$ As; 374 $mg \cdot kg^{-1}$ Cu; 1098 $mg \cdot kg^{-1}$ Mn; 2347 $mg \cdot kg^{-1}$ Pb and 633 $mg \cdot kg^{-1}$ Zn. *Cistus ladanifer* subsp. *ladanifer* has naturally colonised this tailing and now it is the dominant species. (Photo: C. Quintela-Sabarís)

CHAPTER 4

Heavy metal accumulation in leaves of divergent chloroplast lineages of the pseudometallophyte *Cistus ladanifer* L. Implications for phytostabilization

Keywords: Accumulation, *Cistus ladanifer*, heavy metals, pseudometallophyte

ABSTRACT

Cistus ladanifer is a shrub which grows in different kinds of soils (including serpentine outcrops and human-polluted sites) in the Western Mediterranean area. Chloroplast DNA analyses have inferred that metallicolous populations of this plant have arisen through distinct foundation events within two different postglacial recolonisation lineages.

We have quantified the levels of Co, Cr, Cu, Mn, Ni, Pb and Zn in soils and in leaves of *C. ladanifer* plants from 33 populations, covering almost its entire natural range. In addition, we have computed the ratio between metal contents in leaves and in soils, as a measure of accumulation abilities. Through a nested analysis of variance (nANOVA) we tried to evaluate whether the population type (metallicolous vs. non-metallicolous) or the chloroplast lineage ('North' vs. 'South') differ in their metal contents and accumulation patterns.

Our results show that, on a broad scale, metallicolous populations have higher Ni contents than non-metallicolous ones ($p<0.001$) and lineage 'North' higher Mn contents than lineage 'South' ($p<0.001$). In addition, Cistus ladanifer clearly rejects the accumulation of Co, Cr and Pb in its leaves, whereas for the other metals there were different accumulation patterns between lineages and population types.

The implications of our results in the use of *Cistus ladanifer* for phytostabilization procedures are discussed.

4.1 Introduction

Areas with high contents of heavy-metals in soils are the result of natural processes (weathering of ultramafic rocks) or human activities (mining and industrial activities, atmospheric deposition, excessive use of agrochemicals or even traffic emissions).

Several technologies have been employed in order to remediate human polluted soils, the most of them based on expensive mechanical soil treatments that sometimes include soil removal and replacement. In recent years, phytoremediation, that is, the use of different plant species for soil remediation, has been proposed as an environmentally friendly technology that in addition has a lower economic cost than traditional approaches (Padmavathiamma and Li 2007).

Plant growth is inhibited in the most severely contaminated sites, so human and animal exposure to heavy metals can be increased by the migration of contaminated soil (erosion, dispersal by wind) or leaching into groundwater (Ruttens *et al.* 2006).

In sites with high and multi-elemental contamination, phytostabilization (the use of native or introduced plants to transform soil metals to less toxic forms, but not remove the metal from the soil, Chaney

Table 4.1: Description of *Cistus ladanifer* populations included in this work.

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with dispersed *Pinus pinea* and *P. halepensis* trees	4.79º W	36.84º N
Almodóvar (EAL)	North	M (h)	Clays close to a road	Grassland with dispersed *C. ladanifer* plants	5.65º W	36.16º N
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N
Despeñaperros (EDE)	North	M (h)	Quartzites, close to the highway.	Dense *Cistus ladanifer* shrubland with *Quercus ilex* and *Juniperus oxycedrus*	3.51º W	38.39º N
Fuente Saúco (EFS)	North	NM	Sandstone and conglomerates	Shrubland with *Quercus ilex*	5.51º W	41.19º N
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N
Grazalema (EGR)	South	NM	Decarbonated limestone	Shrubland with *C. ladanifer* and *C. monspelliensis*	5.27º W	36.78º N
Guadarrama (EGU)	North	NM	Granite	Dense shrubland with isolated *Quercus ilex*	4.10º W	40.68º N
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N
Valdecaballeros (EVC)	North	NM	Sedimentary material (gravels, clays)	Dense shrubland with *Pinus pinaster*	5.34º W	39.33º N
Bni Hadifa (MBH)	South	NM	Sandstones	Open *Pinus halepensis* forest	4.17º W	35.02º N
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N
El Jebha (MEJ)	South	NM	Sandstone	Open *Pinus halepensis* forest	4.64º W	35.18º N
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks	4.64º W	34.95º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

Table 4.1: (continued)

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89º W	35.29º N
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N
Tanger (MTA)	South	NM	Sandstone	Open *Pinus pinaster* forest	5.93º W	35.78º N
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18º W	37.88º N
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87º W	41.85º N
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78º W	37.07º N
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03º W	37.39º N
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82º W	41.52º N
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75º W	41.78º N
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98º W	37.03º N
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29º W	40.43º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

et al. 1997) is the most suitable method of remediation (Kidd *et al.* 2009).

As the plants cover the soil surface, they prevent erosion, reduce water percolation and increase biodiversity (Brown *et al.* 2005). In addition, the biological activities and the production of organic matter by the vegetation may contribute to metal immobilization (Vangrosveld *et al.* 2009).

Identification and characterization of plant species capable of growing and surviving in polluted areas could be very helpful in developing phytostabilization technologies. Ideal plant species should have a rapid growth rate and dense root and shoot systems. Moreover, this species should not accumulate metals into above-ground tissues to prevent wildlife exposure and surface contamination.

In addition, the plants used in phytostabilization work in the Mediterranean region should be adapted to water stress in order to cope with a long dry summer season (Frérot *et al.* 2006). Given the fact that the introduction of alien (and potentially) invasive species could produce detrimental effects on the surrounding ecosystems (Méndez and Maier, 2008; and references therein), the use of native plants for local flora in phytostabilization procedures should be a priority.

Cistus ladanifer L. (Cistaceae) is a woody, semideciduous shrub found growing in a wide range of latitudes, altitudes, climatic conditions and soil types in the Western Mediterranean region. Its populations constitute early successional stages adapted to disturbances operating in Mediterranean ecosystems, especially fire (Bastida and Talavera 2002). Moreover, this species retains potentially active leaves through the summer drought (Núñez-Olivera *et al.* 1996). It has been found that natural populations of this plant can produce up to 4,600 kg of dry matter $ha^{-1}.year^{-1}$ of litterfall that improves soil quality (Simões *et al.* 2009). In addition, it develops dense root and shoot systems that can limit the erosion of soil (Martín Bolaños and Guinea López 1949).

This species is a pseudometallophyte. Thus, it is present in non-metalliferous soils and also in metalliferous areas (ultramafic outcrops and also mine tailings) from the North of Morocco to NW of the Iberian Peninsula (Alvarenga *et al.* 2004, Ater *et al.* 2000, Batista 2003, Díez-Lázaro *et al.* 2004, Freitas *et al.* 2004, Pratas *et al.* 2005). In these sites, *C. ladanifer* behaves as an indicator, or even an accumulator, of different heavy metals. In previous work using chloroplast DNA markers, (Quintela-Sabarís *et al.* 2010) we inferred that metallicolous populations of this plant have arisen through distinct foundation events within two different postglacial recolonisation lineages.

Given the fact that variations in tolerance and accumulation capacity are genetically controlled, metallicolous populations with independent origins might show different patterns of response to heavy metals, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Cerastium alpinum* (Nyberg Berglund *et al.* 2003) or *Thlaspi caerulescens* (Assunção *et al.* 2003).

The aim of this study was to analyse the Co, Cr, Cu, Mn, Ni, Pb and Zn leaf contents of field-collected plants from numerous metallicolous and non-metallicolous populations of *C. ladanifer* from two diverging chloroplast lineages in order to explore the extent and variability of metal accumulation in natural populations of the species and thus determine if any populations are more useful for phytostabilization procedures. To compare the metal accumulation abilities of plants originating from metalliferous and non-metalliferous soils, ratios between metal content in leaves and in soils were calculated (Bert *et al.* 2002). More precisely, the following questions were addressed: (i) Do the metallicolous (M) and non-metallicolous (NM) populations differ in metal accumulation? and (ii) Do the M and NM populations from different chloroplast lineages show differences in metal accumulation patterns?

Table 4.2: Heavy metal contents in leaves of *Cistus ladanifer*. The mean ± SE is presented for each population. Last three rows indicate the overall mean, maximum and minimum contents measured in the leaves. Values are expressed in $\mu g.g^{-1}$ of dry weight. Population codes in **bold type** indicate those which we considered as metallicolous (M). Population codes are the same than Table 4.1.

Pop	Co	Cr	Cu	Mn	Ni	Pb	Zn
EAC	1.6 ± 0.9	24.1 ± 5.6	10.7 ± 2.4	60.5 ± 7.7	36.9 ± 5.2	1.2 ± 0.8	75.2 ± 9.8
EAL	5.5 ± 4.5	28.8 ± 9.2	13.4 ± 2.9	734.1 ± 544.2	20.0 ± 5.4	0.4 ± 0.5	123.5 ± 27.7
EBE	5.8 ± 4.5	23.1 ± 3.4	11.0 ± 2.3	316.3 ± 107.6	11.8 ± 1.9	0.5 ± 0.4	89.8 ± 31.2
ECA	2.3 ± 0.9	25.5 ± 12.3	8.9 ± 2.1	539.5 ± 323.9	10.2 ± 2.9	0.9 ± 0.4	105.5 ± 107.9
ECO	2.8 ± 2.1	25.5 ± 12.2	12.4 ± 3.7	1688.5 ± 1324.6	19.3 ± 5.9	4.2 ± 2.3	88.2 ± 15.0
ECR	2.4 ± 2.0	23.0 ± 4.2	13.3 ± 3.6	1078.1 ± 799.6	17.1 ± 5.5	0.1 ± 0.1	86.1 ± 9.1
EDE	5.7 ± 2.7	24.9 ± 8.1	14.9 ± 3.4	865.1 ± 253.2	15.9 ± 2.9	0.7 ± 0.5	97.1 ± 18.0
EFS	3.7 ± 3.0	21.1 ± 5.2	15.5 ± 4.4	1135.4 ± 374.9	32.3 ± 2.8	0.2 ± 0.3	122.1 ± 36.5
EGJ	3.3 ± 1.6	21.7 ± 2.7	11.1 ± 1.7	914.6 ± 462.6	18.6 ± 4.6	1.2 ± 0.4	95.4 ± 9.7
EGR	0.8 ± 0.3	22.7 ± 3.3	14.9 ± 4.2	310.4 ± 100.5	17.8 ± 4.2	0.1 ± 0.1	100.3 ± 15.7
EGU	0.7 ± 0.4	20.8 ± 3.1	10.8 ± 2.4	523.8 ± 257.4	10.2 ± 2.8	0.5 ± 0.8	102.3 ± 20.2
EMA	2.6 ± 1.6	28.4 ± 9.2	37.4 ± 6.9	264.3 ± 88.5	14.8 ± 1.7	0.7 ± 0.6	128.7 ± 33.9
EMF	2.5 ± 0.6	25.8 ± 4.1	12.8 ± 4.4	344.5 ± 235.8	18.0 ± 4.8	0.3 ± 0.3	93.9 ± 26.4
ERB	4.7 ± 1.0	29.4 ± 8.5	8.3 ± 0.8	483.0 ± 149.5	18.0 ± 2.7	0.2 ± 0.4	143.4 ± 86.0
ESB	7.8 ± 8.9	29.3 ± 11.0	8.5 ± 1.7	275.6 ± 331.9	50.0 ± 16.6	0.3 ± 0.3	79.0 ± 30.7
ESP	1.5 ± 0.8	31.2 ± 7.1	8.3 ± 1.3	117.5 ± 39.9	58.8 ± 17.9	0.1 ± 0.2	76.6 ± 14.3
ETO	2.0 ± 0.7	27.3 ± 7.3	6.6 ± 0.9	98.7 ± 14.2	75.7 ± 7.5	0.3 ± 0.3	63.3 ± 6.6
EVC	2.3 ± 1.3	30.2 ± 6.5	8.6 ± 2.3	805.2 ± 343.9	14.4 ± 3.1	0.3 ± 0.3	108.6 ± 32.7
MBH	3.2 ± 1.7	23.8 ± 5.3	13.5 ± 3.8	336.9 ± 80.2	12.6 ± 2.0	0.3 ± 0.4	86.3 ± 19.1
MBT	3.8 ± 1.6	25.4 ± 4.0	10.5 ± 3.2	1179.0 ± 573.4	18.7 ± 4.4	0.4 ± 0.4	93.5 ± 28.1
MEJ	1.5 ± 1.1	30.8 ± 15.4	8.5 ± 0.5	264.0 ± 148.1	12.5 ± 4.6	0.1 ± 0.2	92.7 ± 13.8
MKE	4.7 ± 2.5	25.5 ± 6.4	12.6 ± 2.8	554.9 ± 120.1	15.7 ± 2.5	0.2 ± 0.2	85.6 ± 20.8
MSI	1.0 ± 0.7	26.2 ± 7.6	8.2 ± 1.1	51.6 ± 27.4	47.1 ± 6.8	0.5 ± 0.5	66.1 ± 9.1
MSII	1.2 ± 0.2	25.3 ± 3.8	7.3 ± 1.1	59.3 ± 25.3	53.7 ± 12.8	0.5 ± 0.4	73.8 ± 8.3
MTA	1.5 ± 0.7	25.2 ± 4.8	12.5 ± 4.1	78.8 ± 37.5	10.9 ± 1.9	0.2 ± 0.2	83.9 ± 27.7
PAL	6.7 ± 2.4	27.2 ± 8.0	26.6 ± 3.2	980.7 ± 385.0	25.7 ± 6.4	1.7 ± 0.9	288.0 ± 94.5
PBR	2.1 ± 1.5	25.7 ± 4.0	9.3 ± 2.7	232.6 ± 43.5	74.5 ± 13.8	0.3 ± 0.4	85.1 ± 16.2v
PBU	0.6 ± 0.5	23.3 ± 4.9	9.8 ± 2.5	174.5 ± 169.7	8.3 ± 1.4	0.4 ± 0.4	56.8 ± 14.6
PCF	5.4 ± 2.5	22.4 ± 6.5	12.2 ± 2.9	1485.8 ± 414.8	18.7 ± 2.9	0.2 ± 0.2	57.5 ± 8.0
PMC	2.6 ± 2.0	24.7 ± 7.1	12.5 ± 1.4	308.1 ± 338.6	99.6 ± 41.6	0.2 ± 0.3	87.0 ± 9.5
PSA	1.5 ± 0.3	50.0 ± 9.1	8.1 ± 0.8	186.2 ± 62.2	93.1 ± 28.3	0.3 ± 0.3	77.5 ± 18.4
PSV	0.3 ± 0.2	24.5 ± 5.2	10.8 ± 2.2	52.4 ± 15.2	9.7 ± 2.4	0.3 ± 0.3	78.2 ± 34.1
PVE	1.9 ± 1.1	20.0 ± 4.7	9.6 ± 2.3	809.6 ± 518.3	10.2 ± 2.3	0.1 ± 0.1	71.9 ± 20.8
Overall Mean	2.9	26.2	12.1	517.1	29.4	0.5	95.7
Max.	25.1	61.12	46.13	3952.3	141.3	8.1	423.7
Min.	0.03	11.32	4.43	20.71	6.1	0	35.2

4.2 Material and methods

4.2.1 Study species

Cistus ladanifer is an entomophyllous, obligatory outcrossing species, bearing a gametophytic mechanism of incompatibility (Talavera *et al.* 1993). It is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). Three subspecies have been described based on leaf traits (Demoly and Montserrat 1993). Two subspecies, *Cistus ladanifer* subsp. *ladanifer* and subsp. *africanus*, are widespread and they have colonized metalliferous (ultramafic) areas, although only subsp. *ladanifer* is found also in mine tailings from the Iberian Peninsula. Finally *C. ladanifer* subsp. *sulcatus* (formerly *C. palhinhae*) is restricted to limestone-derived soils on the coast of the south-western tip of Portugal.

4.2.2 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire natural range of this species (and its three subspecies) were sampled. In previous work (Quintela-Sabarís *et al.* 2010), on the basis of soil metal contents (either Total or Ammonium Acetate/Acetic Acid/EDTA extractable contents) we have classified these populations as metallicolous (M) and non-metallicolous (NM). M populations included those growing on ultramafic outcrops in Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), or on human-polluted soils, such as mine tailings, or areas affected by highway traffic.

In addition, using chloroplast microsatellites (cpSSRs) we have inferred that M and NM populations belong to two independent lineages ('North' and 'South') that were isolated during last Glacial period (Quintela-Sabarís *et al.* 2010).

In each population, a longitudinal transect was established, along which six plants, separated by at least 5m, were selected. We collected 5 branches with leaves from each of the selected plants. At the laboratory, leaves were separated from stems, and they were cleaned with fresh water, rinsed twice with distilled-deionised water and dried at 60ºC for two days. In addition, in each site soil samples were collected from 5-15 cm in depth. Each soil sample was air-dried and sieved through a 2mm-mesh.

4.2.3 Chemical analyses

Dried leaves and soil samples were ground to achieve homogeneity. Soil Total and extractable metal contents were quantified previously (Quintela-Sabarís *et al.* 2010).

The amounts of Cr, Cu, Mn, Ni, Pb and Zn in leaves were quantified in solid subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF). Other subsamples were digested with HNO_3 for the quantification of Co contents with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) element analysis.

Any value lower than the detection limit was recorded as 0.1 $\mu g \bullet g^{-1}$ for statistical analyses (Bert *et al.* 2002).

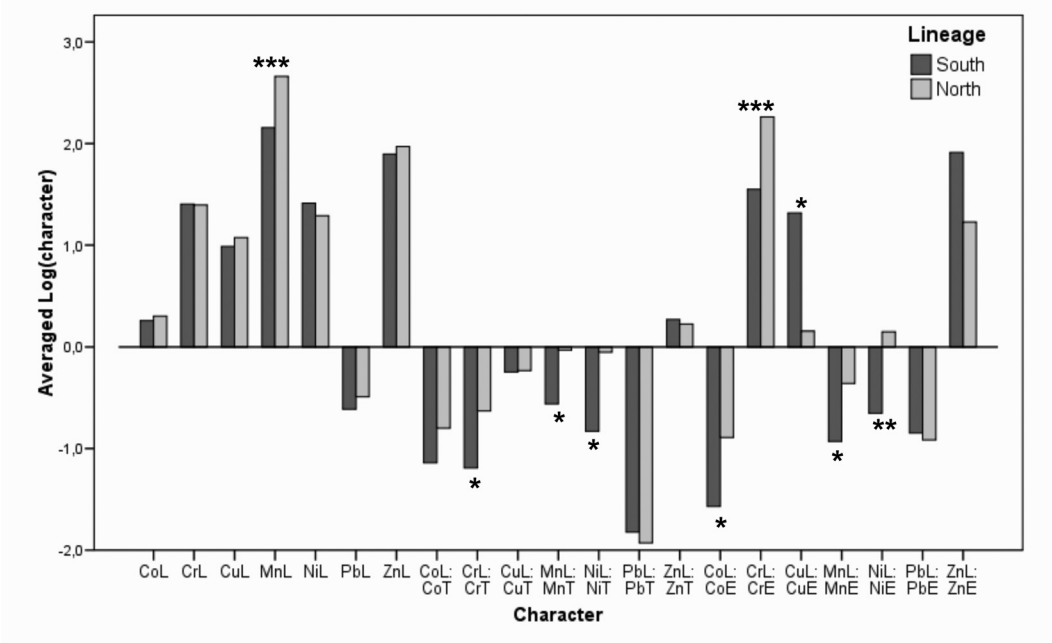

Figure 4.1: ANOVA results for the factor 'Lineage'. Asterisks indicate statistically significant differences. * $P < 0.05$; ** $P<0.01$; *** $P<0.001$. Means for each group were log-transformed for graphical purposes.

4.2.4 Statistical analyses

In a previous study we obtained the total soil contents (CoT, CrT, CuT, MnT, NiT, PbT, ZnT) and extractable soil contents (CoE, CrE, CuE, MnE, NiE, PbE, ZnE) (Quintela-Sabarís *et al.* 2010) (Soil data are available in Supplementary Material S 3.1).

To test whether metal contents in plants differed between plant populations according to their chloroplast lineage and their type (metallicolous or non-metallicolous), a nested analysis of variance (nANOVA) type III SS was performed on the basis of the leaf contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn. These variables were represented as CoL, CrL, CuL, MnL, NiL, PbL and ZnL.

In addition to these 7 variables, the ratio of the metal concentration in leaves over the total and the extractable metal concentration in soils was calculated in order to compare metallicolous and nonmetallicolous populations for the amount of accumulation of these heavy metals. Thus, 14 new variables were created, leading to a total of 21 characters.

All variables were transformed using a BOXCOX transformation in order to improve the fit to a normal distribution of the data. In most cases, BOXCOX yielded a value of power parameter λ equal to 0, so most variables were log-transformed.

The nANOVA model was Lineage, Type, interaction Lineage × Type and population within the interaction Lineage

× Type. Lineage ('North' vs. 'South'; see Quintela-Sabarís *et al.* 2010) and Type (Metallicolous vs. Non-Metallicolous) were fixed effects, whereas Population was included as a random effect.

For the ratios of metals in leaves over extractable metals in soils, only the variation among metalliferous soils could be analysed, because of the very low extractable concentrations of heavy metals found in non-metalliferous soils. In these cases, the nANOVA model was Lineage and Population within Lineage.

Moreover, the relationship among metals in leaves and soils was evaluated using the non-parametric Spearman's Correlation Coefficient. We chose this test since it does not require assumptions of normality of data.

A Principal Component Analyses (PCA) was performed on means of leaf metal contents for each population. A Varimax rotation was used in order to make the interpretation of the PCs easier. Through the PCA, we summarized the results of the analysis of variance in order to confirm whether plant populations are grouped according to their lineage or type.

All computations were performed with SPSS 15.0 for windows, except the BOXCOX transformations, for which we used the MINITAB 15.1 software.

4.3 Results

The mean contents for each metal in each population are presented in Table 4.2. Except for Cu, the higher metal contents were quantified in leaves from M populations. The case of Mn is especially interesting, given the fact that 37% of plants analysed showed more than 500 $\mu g.g^{-1}$, and 16% more than 1000 $\mu g.g^{-1}$, almost reaching 4000 $\mu g.g^{-1}$ in one plant from population ECO. In addition, 59 plants (30% of total sample) have Zn contents above 100 $\mu g.g^{-1}$.

The Spearman correlation coefficient revealed that for two metals (Co and Mn) the concentrations in leaves are not related to the concentrations in soils (neither total nor extractable) (Table 4.3). The contents of Cr, Ni and Pb in leaves were correlated with total and extractable metals in soils, although the correlation was better with total soil contents than with extractable metals. In the case of Cu and Zn, significant correlations were only found between the metals in leaves and the extractable metals in soils (Table 4.3). CoL and MnL were negatively correlated to soil pH (in acid soils-lower pH-, higher values), whereas NiL was positively correlated to soil pH (at higher pH, higher Ni contents) (Table 4.3).

The ANOVA analyses revealed that, irrespective of the population Type (M or NM), the plants from lineage 'North' showed significant higher values than lineage 'South' for the characters MnL and the ratios CrL:CrT, MnL:MnT, NiL:NiT. In addition, in the case of the ratio leaves:soil extractable, the M populations from lineage 'North' showed higher values of CoL:CoE, CrL:CrE, MnL:MnE and NiL:NiE than M populations from lineage 'South'; whereas CuL:CuE was significantly higher in M populations from lineage 'South' (Fig. 4.1).

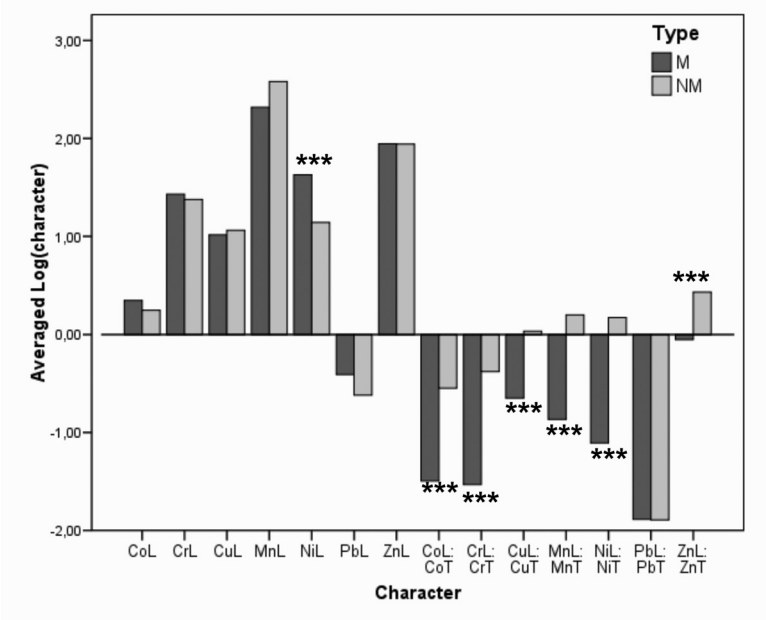

Figure 4.2: ANOVA results for the factor 'Type'. Asterisks indicate statistically significant differences. * $P < 0.05$; ** $P<0.01$; *** $P<0.001$. Means for each group were log-transformed for graphical purposes.

Taking into account the factor Type, significant differences between M and NM populations of Ni content in leaves were found (Fig. 4.2). In addition, the ratios leaf: soil total for all the metals, except Pb, were significantly higher in NM populations.

The interaction Lineage × Type, revealed significant differences only for the characters ZnL and CoL:CoT (Fig. 4.3). ZnL in M populations of lineage 'North' was higher than NM populations from that lineage, whereas in lineage 'South' the M populations had a ZnL value lower than NM ones.

In the case of CoL:CoT, the group of M populations have lower values than NM populations for both lineages.

However, the accumulation of metals varied considerably degree among populations, since for all the characters analysed the factor 'Population' nested within the interaction Lineage × Type showed significant values (in CrL, $P < 0.01$; in the remaining characters, $P < 0.001$).

The combined analysis of the quantities of metals in leaves resulted in a PCA where the M populations from human-polluted sites are placed next to other NM populations along PC1 (which is mainly made up of ZnL, CuL and CoL), whereas the M populations from ultramafic areas are placed apart from the other populations, with negative scores on the first PC and positive scores on the second PC (mainly made up of CrL and NiL) (Fig. 4.4a).

When considering all the ratios leaf: total soil, M populations (with ratio values usually lower than, or near, 1) are grouped in the negative scores on PC1. In contrast, NM populations are more scat-

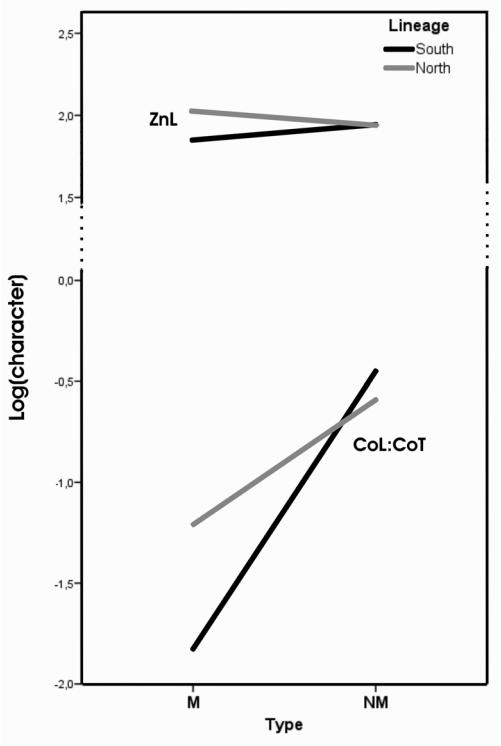

Figure 4.3: ANOVA results for the interaction 'Lineage × Type'. Only traits that showed significant differences among groups were presented. In both cases, $P < 0.05$. Means for each group were log-transformed for graphical purposes.

tered along the two axes (Fig. 4.4b).

In both cases, due to its greater diversity (ultramafic areas and also human polluted sites) M populations from lineage North are more dispersed along the graphics than M populations from lineage South (serpentine outcrops only).

4.4 Discussion

In this work we report the results of a broad scale analysis of the pseudometallophytic shrub species *Cistus ladanifer*. We tried to integrate phylogeographic information about this plant into the analysis of its patterns of heavy metal accumulation in leaves.

We have found that this plant accumulates quantities of metals that in some cases(such as Cr, Mn, Ni, Zn) are above the described critical concentrations in plants (Kabata-Pendias 2001) that is, *C. ladanifer* stores, on average, potentially toxic contents of Cr, Mn and Ni in its leaves. Moreover, the levels of Zn in one third of plants analysed are higher than critical concentrations.

Our data fall within the range of results obtained in previous studies of *C. ladanifer* at local and regional scales (Pratas 1996, Alados *et al.* 1999, Ater *et al.* 2000, Batista 2003, Alvarenga *et al.* 2004, Freitas *et al.* 2004, Díez-Lázaro *et al.* 2006), but for the metals Co, Mn and Ni we have quantified values in leaves that exceed (and in the case of Mn and Ni even double) values reported in the literature

However, our sampling took place in mid summer, after the maximum litterfall of *C. ladanifer* (before summer drought, Núñez-Olivera *et al.* 1993) so the differences between our data and others can be justified by seasonal variations in growth and relative metal contents. Along these lines, the maximum quantities of Sb of *C. ladanifer* were measured in autumn-collected leaves (Murciego Murciego *et al.* 2007).

Through ANOVA analyses, we have found significant effects of the factors Lineage, Type, and Lineage × Type for the quantities of the metals Mn, Ni and Zn, respectively.

All these three elements are micronutrients essential to plant metabolism (Rengel 2004, Epstein and Bloom 2005, and references therein), although their behaviour and functions are different. The foliar concentration of Ni was correlated with soil concentrations. Thus, the M populations (the majority of which are growing in Ni-rich serpentine outcrops) have significantly more foliar Ni than NM ones. This is the general situation in other plant species (Brooks 1987: pp 38).

In the case of foliar Mn, the lineage 'North' possessed significantly higher foliar contents than lineage 'South'. With our data, this difference can not be explained by metal contents in soils (total or available), although a low but significant correlation was observed between MnL and soil pH. However, it seems that soil pH alone cannot explain the higher MnL in lineage North, since ANOVA analyses showed no significant differences in soil pH between lineages (data not shown).

In their work with populations of *C. ladanifer* growing on and around a mine tailing, Alvarenga *et al.* (1999) suggested a relationship between Mn accumulation in leaves and intensity and duration of exposition to sunshine. Due to the broad scale of our work, we do not have evidence to support this suggestion. However, high contents of Mn were observed in populations from the C of the Iberian Peninsula (Lineage 'North'), an area with high summer temperatures, so more investigation would be necessary in order to clarify this topic.

The foliar levels of Zn are influenced by the interaction of factors Lineage and Type. The NM populations of both lineages showed similar levels of foliar Zn, whereas the foliar Zn is higher in M populations from lineage 'North' than M populations from lineage 'South'. This interaction could be caused by the fact that, in our work, human polluted sites (traffic, mine tailings), which presented the higher quantities of Zn both in leaves and soils, are found only in lineage 'North', whereas in lineage 'South' the M populations are all from serpentine outcrops with lower Zn contents.

Baker (1981) defined three basic strategies of metal uptake by plants. Following these definitions, which are based on the ratio between aerial plant part over total metal content in soil, we have observed that *C. ladanifer* behaves as a excluder of Co, Cr and Pb (ratios leaves: soil lower than 1 irrespectively of population type or chloroplast lineage), whereas the accumulation rates of Cu, Mn, Ni and Zn in leaves differs significantly between Lineages and also between population Types.

More specifically, NM populations usually had higher accumulation rates than M ones, under the conditions of our study (growing in non-metalliferous soils). Given this fact, the total amounts of heavy metals (except Ni) in leaves were similar in both types of populations. This difference in accumulation was observed in other pseudometallophytes such as *Arrhenatherum elatius* (Deram *et al.* 2007), *Silene vulgaris* (Chardonnens *et al.* 1998) and the hyperaccumulator *Arabidopsis halleri* (Bert *et al.* 2000), and even in a

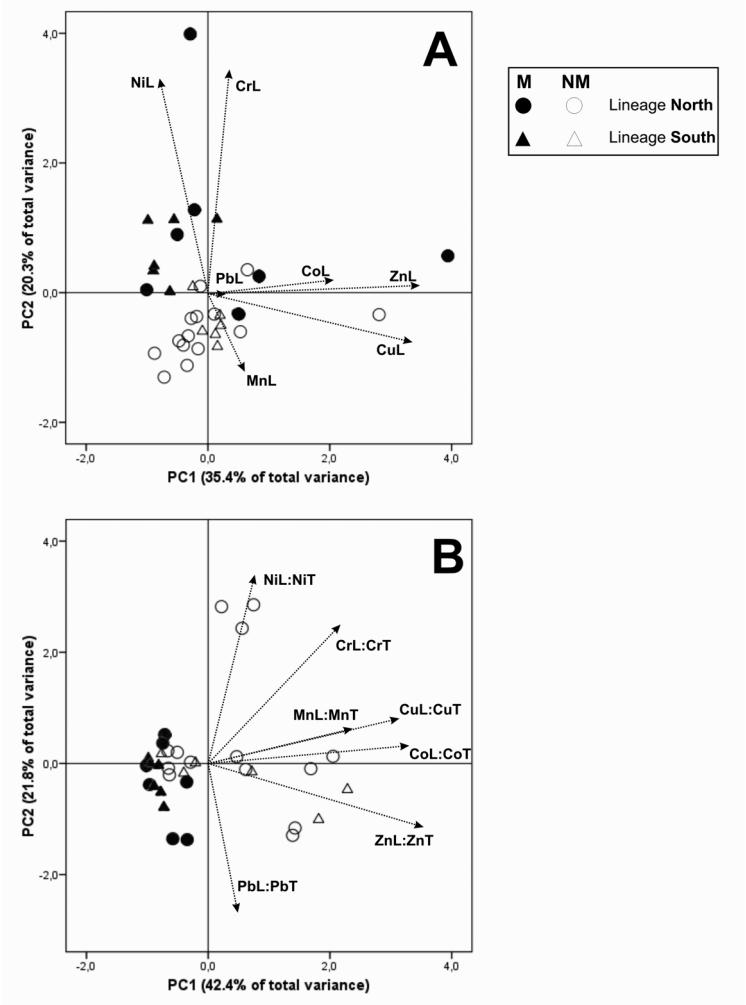

Figure 4.4: Principal Component Analyses (PCA) based on **A**: leaf contents (averaged for each population) of Co, Cr, Cu, Mn, Ni, Pb and Zn; **B**: ratios of leaf and Total soil contents of Co, Cr, Cu, Mn, Ni, Pb and Zn (averaged for each population).
In both cases, filled symbols indicate M populations, whereas empty symbols indicate NM populations. Arrows indicate the contribution of each variable to each of the Principal Components.

Table 4.3: Relationship of metals in *C. ladanifer* leaves with metals in soils and pH. Data show the values of the non-parametric Spearman rho correlation coefficient. Only significant correlations ($P < 0.05$) are presented.

		Metal in Leaves						
		Co	**Cr**	**Cu**	**Mn**	**Ni**	**Pb**	**Zn**
Metal in Soils	**Total**		0.24***			0.73***	0.28***	
	Extractable		0.21**	0.30***		0.70***	0.18*	0.31***
pH		-0.34***			-0.31***	0.20**		

* P<0.05; ** P<0.01; *** P<0.001

work with populations of *Cistus ladanifer* from NE Portugal (Díez-Lázaro *et al.* 2006, Kidd *et al.* 2004). However, most of these studies were all based in hydroponic cultures with controlled metal contents, so we have to be cautious when comparing our results with those.

Deram *et al.* (2007) proposed two explanations for the higher accumulation in NM populations. On the one side, NM populations could develop heavy metal active cellular mechanisms involved in the detoxification of heavy metals. On the other side, M populations could restrict better metal accumulation in aerial parts. Kidd *et al.* (2004) found that NM populations of *C. ladanifer* with low tolerance to Co accumulate more than 3,000 $\mu g.g^{-1}$ Co in shoots but they showed toxicity symptoms when subjected to a 500µM Co treatment; in contrast, the populations with more tolerance to Cu or Ni in hydroponic cultures also showed a low accumulation in aerial parts. Therefore, it appears that in the case of *C. ladanifer*, the most suitable explanation is that of restricted metal accumulation in M populations.

However, there were also differences among M populations. If we consider the ratios computed with extractable metals, which should be more realistically related to plant accumulation abilities, we found that M populations from lineage 'North' showed greater accumulation rates than 'South' for the metals Co, Cr, Mn and specially Ni; whereas M populations from lineage 'South' possessed a greater accumulation rate for Cu.

This phenotypic diversity can be viewed as a result of multiple and independent events of colonisation in areas with heavy metals within diverging chloroplast lineages (Quintela-Sabarís *et al.* 2010). Similarly, serpentine populations of *Cerastium alpinum*, which originated within different recolonisation lineages, developed different growth responses to Ni and Mg (Nyberg-Berglund *et al.* 2004).

The ideal plant species for use in phytostabilisation procedures in an area like the Mediterranean region should be a native plant tolerant to both drought and metal stress, and with an extensive root system. In addition, this species should not accumulate metals into above-ground tissues, in order to prevent further transfer into the food chain and thus reduce human or animal access to contaminants (Frérot *et al.* 2006).

Given these requirements and the ample phenotypic diversity we have observed in *Cistus ladanifer*, Metallicolous populations from lineage 'South' are the best suited for use in phytostabilization procedures in soils polluted with Co, Cr, Mn or Ni, whereas the M populations from lineage 'North' are better for use in Cu polluted soils. However, the considerable differences in the response to those metals among populations means that any remediation procedure should be preceded by a survey that allows the characterization of local ecotypes of *Cistus ladanifer* in relation to heavy metals.

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Chapter 5

Effect of population type and chloroplast lineage on the response of *Cistus ladanifer* L. (Cistaceae) to metals: a hydroponic culture analysis

Previous page: Hydroponically-grown plantlets from different populations of *Cistus ladanifer*. They have been just transferred to plastic buckets in order to perform tests of tolerance to heavy metals. (Photo: C. Quintela-Sabarís)

CHAPTER 5

Effect of population type and chloroplast lineage on the response of *Cistus ladanifer* (Cistaceae) to metals: a hydroponic culture analysis

Keywords: Chlorophyll fluorescence, *Cistus ladanifer*, growth rates, heavy metals, hydroponic culture, tolerance

ABSTRACT

Cistus ladanifer is a pseudometallophyte plant whose metallicolous (M) populations belong to two diverging chloroplast lineages.

Previous studies showed that tolerance to heavy metals could be a characteristic of this plant species. However, it is possible that there are differences in tolerance to metals between population types and/or chloroplast lineages. In order to check these issues, twenty five populations of this species were grown in hydroponic cultures and their tolerance to the trace metals Co, Ni and Zn was assessed.

Growth rates, dry weights and photosystem II fluorescence were measured and transformed into measures of relative tolerance. These data were compared using nested Analysis of Variance and correlation analyses.

Co caused reduction in root growth, whereas Ni and Zn influenced root and shoot growth and also photosynthetic performance. In contrast, the relative increase in the number of leaves was not affected by any treatment. In most cases, the tolerance to metals was similar between population types and chloroplast lineages.

This fact indicates that tolerance to heavy metals could be a common trait in this species. Finally, in view of our findings, we suggest interesting lines for future research, such as the preadaptation of *C. ladanifer* to metals or the possible role of root symbioses in tolerance.

5.1 Introduction

Heavy metals have toxic effects on plant metabolism, such as the production of reactive oxygen species, the blocking of essential functional groups in biomolecules and the displacement of essential metal ions (Schutzendubel and Polle 2002), which subsequently produce reduction in growth, variation in photosynthesis or even death.

Due to these toxic effects, soils with high contents of these elements, either of natural or human origin, are areas with low vegetation cover and, in the case of human-polluted sites, can constitute environmental problems due to the leaching of metals into groundwater or the migration of contaminated soil by erosion and/or dispersal by wind (Ruttens *et al.* 2006). Some plant species have developed mechanisms for metal tolerance (e.g. chelation of metals in the cytosol by phytochelatins, (Goldsbrough 2000)) whose objective is to avoid the build-up of toxic concentrations at sensitive sites within the plant cell (Hall 2002).

This tolerance can be ‘constitutive’ (=’species-wide’; i.e. common to all the individuals of a given species) or ‘population-specific’ (i.e. the tolerance to heavy metals have evolved only in certain

populations of a given species) (Pollard *et al.* 2002).

Different research has indicated that tolerance to heavy-metals in plants; i.e., the ability to grow and survive in soils toxic to other plants, is a characteristic governed by a low number of major-genes, which are also modified by a large number of minor-genes (reviewed in Macnair *et al.* (2000)). As a consequence of the genetic determination of this characteristic, a variation in responses to heavy metals is expected and observed within different species (e.g. Zn tolerance in *Arabidopsis halleri*, Pauwels *et al.* (2006)). This variation in responses is clearer in those species whose metallicolous populations have evolved independently, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Cerastium alpinum* (Berglund *et al.* 2004) or *Thlaspi caerulescens* (Assunção *et al.* 2003).

Cistus ladanifer L. (Cistaceae) is a woody, semideciduous shrub from the Western Mediterranean Area (from the South of France to North of Morocco and Algeria) (Demoly and Montserrat 1993). This plant is a pseudometallophyte; thus, it is present in non-metalliferous soils (mainly derived from acid rocks) and it also has colonised metalliferous areas (serpentine outcrops and mine tailings) throughout its distribution area (Alvarenga *et al.* 2004, Ater *et al.* 2000, Batista 2003, Díez Lázaro *et al.* 2006, Freitas *et al.* 2004, Pratas *et al.* 2005). Using cpSSR markers, we have inferred that the metallicolous populations of this species (i.e., those present on metalliferous soils) have arisen within two diverging chloroplast lineages (Quintela-Sabarís *et al.* 2010). On the basis of similar genetic diversity values between metallicolous (M) and non-metallicolous (NM) populations, we proposed that tolerance to heavy metals could be a 'constitutive' character in *C. ladanifer*.

In the present paper, we investigated the tolerance of *C. ladanifer* to the following heavy metals: Co, Ni and Zn, in 25 populations throughout its whole range of distribution. Previous work has assessed the tolerance of *C. ladanifer* to different heavy metals, but only in five populations from NE of Potugal (Kidd *et al.* 2004). Here, we asked whether tolerance to heavy metals is present both in M and NM populations of *C. ladanifer* and whether differences exist in tolerance to these metals between population types (metallicolous vs. non-metallicolous) and/or chloroplast lineages. Specifically, we estimated tolerance by measuring growth, biomass and photochemical efficiency.

5.2 Material and methods

5.2.1 Populations studied

Twenty-five populations of *C. ladanifer* sampled throughout its natural geographic distribution area were included in this work. These populations were classified in two 'soil types': Metallicolous (M) and Non-metallicolous (NM) (see supplementary material S 3.1 and S 5.1), based on previous soil analyses (Quintela-Sabarís *et al.* 2010). M populations include plants growing in serpentine outcrops from Morocco, Portugal and Spain and also populations developed on mine tailings from

Portugal and Spain.

In addition, previous analyses of chloroplast microsatellites (cpSSR) revealed that these populations come from two chloroplast lineages that spread independently throughout the N of Morocco and SE of the Iberian Peninsula ('South' lineage) and the rest of the Iberian Peninsula ('North' lineage) after the Last Glacial Maximum (Quintela-Sabarís *et al.* 2010). (Table 5.1).

In each population, a longitudinal transect was established at random. 10 plants separated by at least five meters were selected along this transect and their ripe fruits were collected.

5.2.2 Plant material and growth conditions

Samples of seeds from each population were subjected to a dry-heating treatment (100ºC for 30 minutes) in order to break the physical dormancy and increase germination percentages (Perez-Garcia 1997). The treated seeds were then sown in Petri dishes with sterilized acid-washed sand and moisturized with distilled water. Seeds were placed in a culture chamber under the following conditions: 16/8 h light/darkness, day/night temperature 20/15 ºC, RH 70%, and PPFD 190 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Four weeks after germination (when the plantlets reached a four-leaf stage), they were carefully transferred to polystyrene sheets suspended in a nutritive solution with low concentrations of trace metals (control solution) at pH 4.5. This solution, optimized for *Cistus ladanifer* by Kidd *et al.* (2004), had the following composition (µM): 2000 $CaCl_2.2H_2O$, 1000 $MgSO_4.7H_2O$, 250 NH_4NO_3, 50 KH_2PO_4, 200 NaOH, 150 KCl, 25 $ZnSO_4.7H_2O$, 20 $MnSO_4.H_2O$, 15 $CuSO_4.5H_2O$, 15 FeEDTA, 10 H_3BO_3, 0.0143 $(NH_4)_6Mo_7O_{24}.4H_2O$.

Stock solutions of 100-(macronutrients) and 1000-strength (micronutrients) were made up and diluted appropriately. Culture solutions were continuously aerated with an aquarium air pump and changed every week. After 42 d in this solution, seedlings of similar size were selected and randomly placed into thirty 6 L-plastic buckets, each holding 10 seedlings. At this stage seedlings were transferred to a greenhouse (20–28 ºC) with natural light, where they continued to grow until the end of the experiment.

5.2.3 Treatment with metals and ecophysiologycal measurements

Due to space limitations, the 25 populations were grouped in batches of five populations each. A total of 10 successive experiments (two for each batch) were carried out, replicating the same growth conditions previously described.

In each batch, five buckets of *C. ladanifer* plants (each containing two replicates for each population) were randomly allocated to one of the following treatments with trace metals: cobalt (Co), nickel (Ni) and zinc (Zn), and three controls. Treatment concentrations and chemical form in which the metal was added were chosen following Kidd *et al.* (2004) (see Table 5.2 for details). Solutions were completely replaced every three days to

Table 5.1: List of *Cistus ladanifer* populations included in our analyses. For each population, the lineage (North, South) and the type (M, NM) are indicated.

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with dispersed *Pinus pinea* and *P. halepensis* trees	4.79º W	36.84º N
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks	4.64º W	34.95º N
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89º W	35.29º N
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18º W	37.88º N
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87º W	41.85º N
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78º W	37.07º N
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03º W	37.39º N
Martinchel (PMA)	North	NM	Sedimentary material (gravels, clays)	Dense *Pinus pinaster* plantation	8.29º W	39.52º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

Table 5.1: (continued)

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82º W	41.52º N
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75º W	41.78º N
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98º W	37.03º N
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29º W	40.43º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

maintain nutrient supply and treatment metal concentrations.

Length of the longest root (L_R), shoot length (L_S) and the number of leaves (N_L) were recorded before plants experienced trace metals (abbreviated as L_{R0}, L_{S0}, N_{L0}) and at harvesting, after 14 days of growth under the different treatments (abbreviated as L_{R14}, L_{S14}, N_{L14}). We obtained three growth measurements (R_E, S_E, N_L) as the differences between variables on days 0 and 14. In addition, harvested plants were dried at 60ºC to constant weight and the dry weight (abbreviated as DW) was measured.

Photochemical efficiency, as estimated by chlorophyll fluorescence, was measured in vivo on three fully expanded leaves at the top of each plant the night before harvesting (day 13), using a pulse-amplitude modulated fluorometer (Mini-Pam, Walz, Effeltrich, Germany) in 15 of the 25 populations included in this study. Measuring light and saturating light pulses (>4000 µmol photons$\cdot m^{-2}\cdot s^{-1}$, 0.8 s pulse length, actinic white light) were applied through a fibre optic probe at a 60º angle relative to the sample and 12 mm from the leaf. The maximum quantum yield of photosystem II (PSII) was assessed from the ratio $F_v/F_m = (F_m - F_0)/F_m$ (see Bolhàr-Nordenkampf *et al.* 1989), where F_0 and F_m are defined as minimal and maximal fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open. This parameter was measured at night, with dark-adapted plants to ensure that all their PSII reaction centres were open. The maximum quantum yield esti-

Table 5.2: Summary of metal treatments and the chemical form in which they were added to nutrient solutions.

Trace metal	Chemical form added	Treatments (μM)			
		Control	Co	Ni	Zn
Co	$CoSO_4.7H_2O$	0	100	0	0
Ni	$NiSO_4.6H_2O$	0	0	250	0
Zn	$ZnSO_4.7H_2O$	25	25	25	250

mates the efficiency of excitation energy capture by open PSII reaction centres (Butler and Kitajima 1975).

In order to eliminate most of variation on responses unrelated to metal treatments, both growth (R_E, S_E, N_L) biomass (DW) and photochemical efficiency measurements were transformed as a percentage of control plants = 100 × (response in metal treatment) / (mean value of the response in the control), following the original proposal of Wilkings (1978) for root elongation.

Thus, we finally obtained five relative measures of metal-tolerance: relative root elongation (R_{RE}), relative shoot elongation (R_{SE}), relative increase of number of leaves (R_{NL}), relative dry weight (R_{DW}) and relative Yield (R_Y).

5.2.4 Statistical analyses

For each metal (Co, Ni and Zn), we tested for differences between metal treatment (metal vs. control), population type (metallicolous vs. non-metallicolous), chloroplast lineages (North vs. South) and among plant populations, on R_{RE}, R_{SE}, R_{NL}, R_{DW} and R_Y. We used a nested analysis of variance (nANOVA) in which metal treatment (Treatment), population type (Type) and chloroplast lineage (Lineage) were fixed factors, and population was a random factor nested within the interaction Lineage×Type. In order to extract the effects of initial plant size from the analysis, the measures L_{R0}, L_{S0} and N_{L0} were introduced in the model as covariates.

Before carrying out statistical analyses, the data were explored in order to i) detect extreme values, which were not included in the analyses; and ii) check the homogeneity of variances and the normality of the data. When necessary, data were transformed in order to meet ANOVA assumptions of homogeneity and normality.

Finally, the relationships between tolerance to each metal and the previous exposition to metals in field were examined through the computing of the non-parametric Spearman's correlation index among R_{RE}, R_{SE}, R_{NL}, R_{DW}, R_Y and the total (CoT, NiT, ZnT) and extractable (CoE, NiE, ZnE) contents of Co, Ni and Zn in the soils from the populations of origin.

Data analyses were conducted in SPSS (v.15, SPSS Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Effects of 'Treatment'

The three metal treatments caused significantly lower R_{RE} values than the controls (*P*: $R_{RE\text{-}Co}$ 0.001, $R_{RE\text{-}Ni} < 0.001$, $R_{RE\text{-}Zn} < 0.001$), whereas R_{NL} showed no differences between metals and controls. For the other three variables, only plants treated with Ni or Zn showed lower values than controls (Fig. 5.1).

5.3.2 Effects of the interaction 'Treatment × Type'

The three variables related to growth (R_{RE}, R_{SE} and R_{NL}) showed no significant differences for the interaction Treatment × Type (*P*: $R_{RE\text{-}Co}$ 0.299, $R_{RE\text{-}Ni}$ 0.974, $R_{RE\text{-}Zn}$ 0.654, $R_{SE\text{-}Co}$ 0.803, $R_{SE\text{-}Ni}$ 0.479, $R_{SE\text{-}Zn}$ 0.321, $R_{NL\text{-}Co}$ 0.207, $R_{NL\text{-}Ni}$ 0.902, $R_{NL\text{-}Zn}$ 0.852). That is, M and NM populations showed the same growth responses to each of the treatments (Fig. 5.2). In contrast, R_Y revealed significant differences in the treatments with Ni and Zn: whereas 'Controls' from M and NM populations showed similar R_Y values, NM populations showed lower yield values than M populations when subjected to treatments with metals (Fig. 5.2; *P*: $R_{Y\text{-}Co}$ 0.461, $R_{Y\text{-}Ni}$ 0.006, $R_{Y\text{-}Zn}$ 0.009).

In addition, R_{DW} revealed significantly different responses in M and NM populations to the treatments 'Control' and 'Zn'. However, the mean for 'Control' plants (either M or NM) was higher than mean values for 'Zn' plants (Fig. 5.2; *P*: $R_{DW\text{-}Co}$ 0.434, $R_{DW\text{-}Ni}$ 0.186, $R_{DW\text{-}Zn}$ 0.010).

5.3.3 Effects of the interaction 'Treatment × Lineage'

When considering the chloroplast lineages, we only found significant differences for the metals Ni and Zn (Fig. 5.3). Plants from lineage 'North' treated with Ni showed a greater reduction in R_{RE} than plants from lineage 'South' (Fig. 5.3; *P*: $R_{RE\text{-}Co}$ 0.851, $R_{RE\text{-}Ni}$ 0.033, $R_{RE\text{-}Zn}$ 0.533). Plants from lineage 'North' had values of R_{DW} similar to the 'controls', whereas the mean R_{DW} of plants from lineage 'South' was around 60% (Fig. 5.3; *P*: $R_{DW\text{-}Co}$ 0.162, $R_{DW\text{-}Ni}$ 0.753, $R_{DW\text{-}Zn}$ 0.021).

In the case of R_Y, 'controls' of lineage 'South' were higher than 'controls' of lineage 'North', whereas metal-treated plants from lineage 'North' were higher than metal-treated plants from lineage

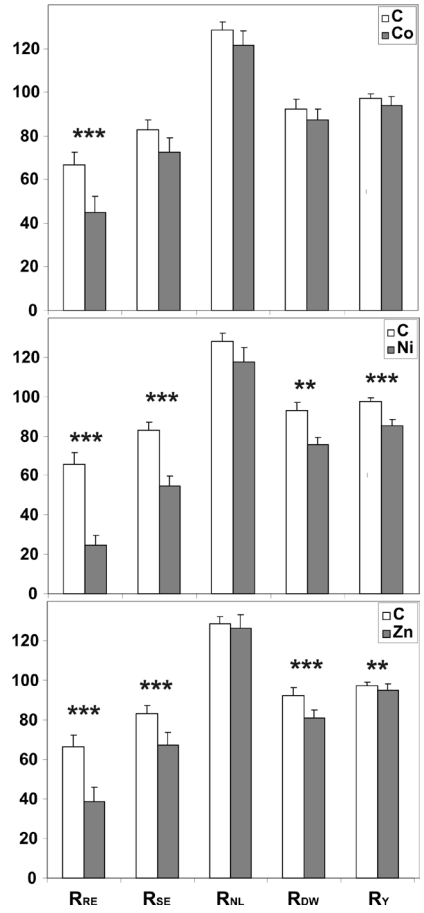

Figure 5.1: nANOVA results for the factor 'Treatment'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001

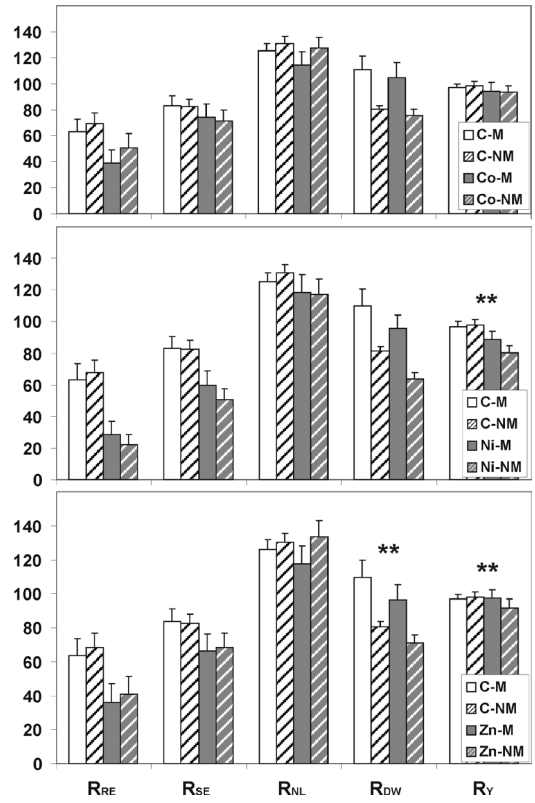

Figure 5.2: nANOVA results for the factor 'Treatment × Type'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

'South'. In other words, when subjected to metal treatments (Ni or Zn) plants from lineage 'South' suffered a greater decrease in their yield than plants from lineage 'North' did (Fig. 5.3; *P*: $R_{Y\text{-}Co}$ 0.941, $R_{Y\text{-}Ni}$ 0.007, $R_{Y\text{-}Zn}$ 0.037).

5.3.4 Other interactions

The interaction 'Treatment × Lineage × Type' was significant only in the case of RDW for the metal Zn (F= 5.56, P = 0.023).

The interaction 'Treatment × Pop(Lineage × Type)', which reflects differences in response to treatments among populations, showed significant differences in the case of the metal Ni for the variables R_{RE} (P = 0.018), R_{SE} (P = 0.049) and R_{DW} ($P < 0.001$).

5.3.5 Correlation tolerance-metals in soils

The values of the Spearman correlation index were non-significant for all the comparisons among metals and tolerance indices (data not shown). Thus, populations from soils with low metal contents show similar (or even higher) tolerance indices than populations from metalliferous areas (Fig. 5.4).

5.4 Discussion

Our work is the first broad-scale assessment of the tolerance of the pseudometallophyte shrub *Cistus ladanifer* to the metals Co, Ni and Zn.

We have shown that each metal caused different toxic effects: Ni and Zn treatments affected root and shoot growth, biomass and photochemical efficiency, Co merely reduced root growth, whereas the number of leaves were not affected by any treatment. Similarly, Kidd *et al.* (2004) also found average reduction in relative root elongation in five populations of *C. ladanifer*, and a greater effect for Ni and Zn than for Co.

Although these three metals have been described in the bibliography as pos-

sessing different critical toxic concentrations (e.g. Kabata-Pendias 2001), we can explain the different effects for each metal on the basis of previous analyses of heavy-metal accumulation that we performed in field-collected leaves from the same populations (data as yet unpublished): we observed that *C. ladanifer* clearly excludes Co and, depending on the chloroplast lineage or the population type, this species behaves as an excluder or an accumulator (*sensu* Baker 1981) of Ni and Zn.

Thus, due to the different behaviour of each metal, we expect Co effects only on roots, as observed, since these organs are the only parts in direct contact with the metal (the plant excludes its transport to aerial parts); however, Ni and Zn can be transported to and accumulated into aerial parts, which may produce alterations of shoot growth and photochemical efficiency, as we indeed detected.

Given the diverse parameters employed by researchers to measure tolerance to heavy metals (growth, survival rates, biomolecules,… for a review see Köhl and Lösch (2004)), and taking into account the previous statement, for future analysis of metal tolerance in plants it would be useful to know the strategy of response to heavy metals in a given species in order to determine the best parameter to be measured.

The significant differences observed for the interaction treatment × lineage point to different mechanisms of response to heavy metals in each independent lineage, as was inferred in *Cerastium alpinum* (Nyberg Berglund *et al.* 2004). However, we have to keep in mind that these differences can be influenced by the fact that lineage North includes M populations from ultramafic areas and mine tailings, whereas the M populations from lineage South are only from ultramafic areas. In fact, the interaction treatment × lineage × type was only significant for R_{DW} for the metal Zn.

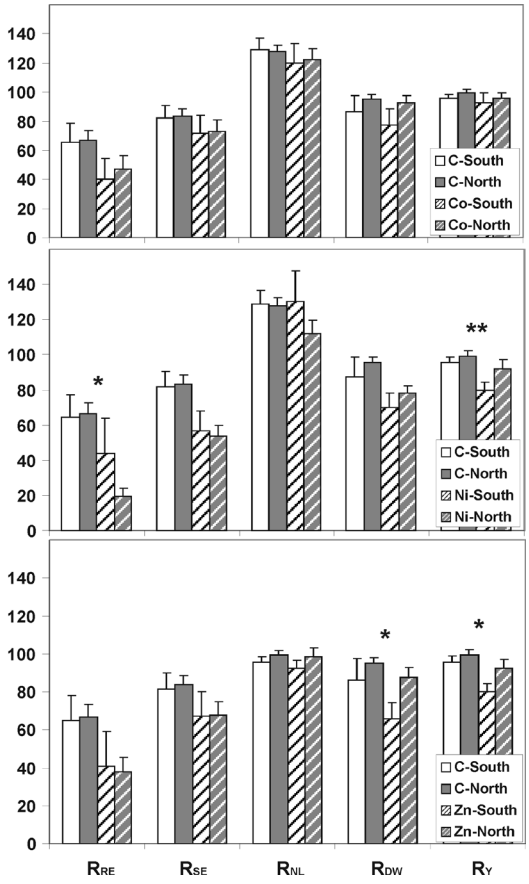

Figure 5.3: nANOVA results for the factor 'Treatment × Lineage'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

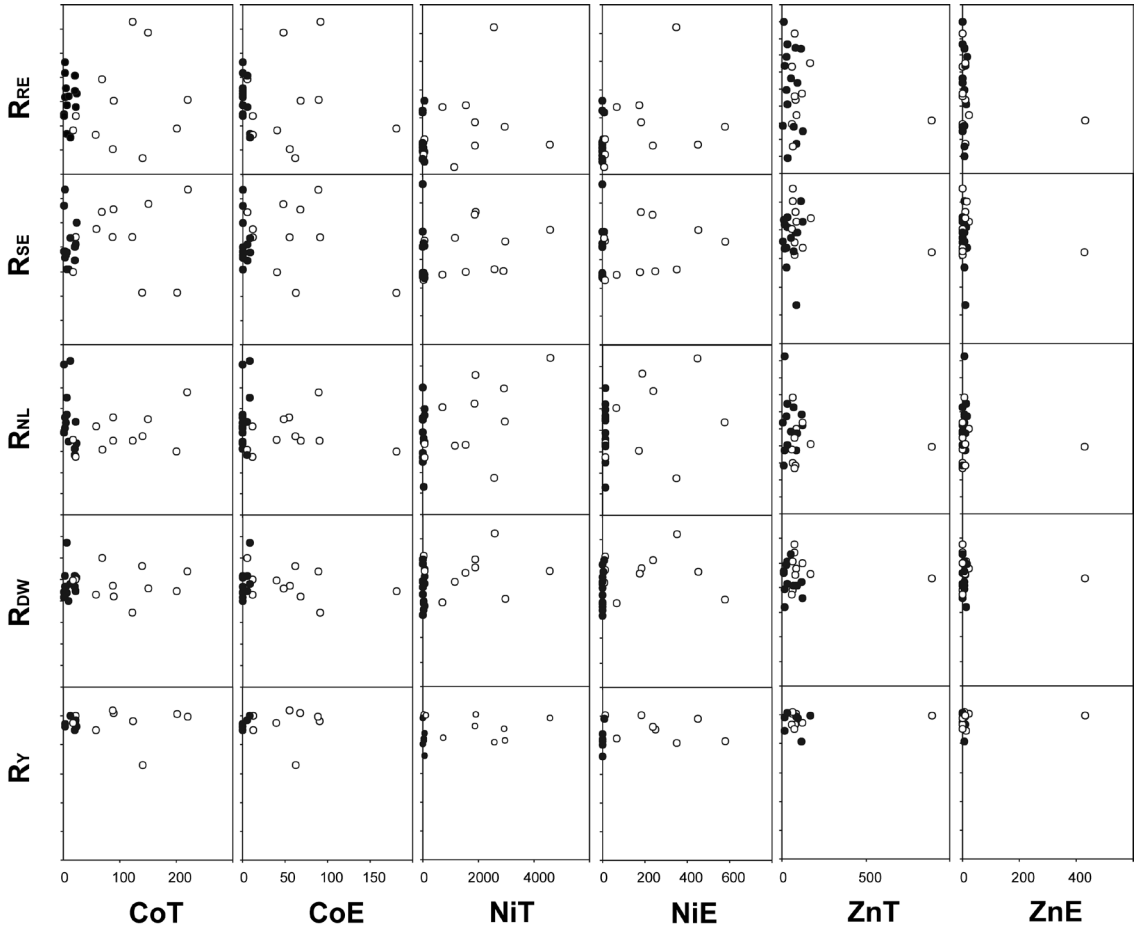

Figure 5.4: Scatterplots of Tolerance Indices *vs.* total (CoT, NiT, ZnT) or Ammonium-acetate/EDTA extractable (CoE, NiE, ZnE) contents of Co, Ni and Zn in the soils of origin. Filled circles indicate NM populations, whereas empty circles indicate M populations.

Based on results previously obtained of the phylogeographic origin and genetic diversity of M populations of *C. ladanifer* (Quintela-Sabarís *et al.* 2010), we proposed tolerance to heavy metals as a constitutive trait in this species. Our results in hydroponic cultures partially support this previous assertion: with the exception of R_{DW} in Zn and R_Y in Ni and Zn, the other measured parameters showed non-significant differences for the interaction treatment × type; whereas the interaction lineage × treatment × type showed significant differences only in the case of R_{DW} and Zn. In addition, populations with low concentrations of metals in the soils of origin showed indices of tolerance similar or even higher than M populations.

We may speculate that findings point to two possible explanations (which are not mutually-exclusive): Firtsly, a preadaptation of *Cistus ladanifer* to soils rich in heavy metals. Macnair (1987) suggested that plant species that are preadapted for any of the harsh conditions of metal rich soils (such as water stress or nutrient shortage) successfully colonize these regions more easily; moreover, Taylor and Levy (2002) detected preadaptation to low Ca:Mg ratios (one of the selective factors of serpentine soils) in a variety of *Phacelia dubia* that was endemic to granite outcrops. In the case of *C. ladanifer*, different authors provide arguments which support the hypothesis of preadaptation: Núñez-Olivera *et al.* (1996) uncovered a high leaf plasticity in *C. ladanifer*, which implies an intrinsic adaptation to scarcity of water and nutrients in this species; Alados *et al.* (1999) suggested that the abilities of *C. ladanifer* inhabiting acidified soils with low Ca concentrations allowed the plants to perform well in serpentine soils in spite of the presence of heavy metals; finally, Kidd *et al.* (2004) observed that plants from non-metallicolous population of *C. ladanifer* maintained high growth rates in presence of Zn in hydroponic cultures.

A second hypothesis that should be explored in future research, partially linked to that of preadaptation, is the possibility that *C. ladanifer* colonised metalliferous areas through collaboration with soil micro-organisms such as fungi and rhizobacteria, given that ectomycorrhizal fungi can provide protection and improve the tolerance to heavy metals in host species (Jentchske *et al.* 2000). Along these lines, Ramos Solano *et al.* (2006) have detected different species of plant-growth promoting rhizobacteria (PGPR) in *C. ladanifer* roots, most of them involved in P-mobilisation and production of siderophores. In addition, more than 30 fungal species have been recorded as establishing symbiotic relations (ectomycorrhiza) with *C. ladanifer* in the literature (Comandini *et al.* 2006). Thus, the negative effects of heavy metals we observed in the plants under metal-treatments could be caused by the lack of these root symbionts in the conditions of hydroponic culture (Epstein and Bloom 2005).

In summary, our investigation revealed that Co, Ni and Zn produced different toxic effects in C. ladanifer plants growing in hydroponics. In most cases the response to metals is similar between population types or chloroplast lineages, a fact that points to the tolerance to heavy metals as a common trait in this species. Moreover, interesting lines for future research, such as the occurrence of preadaptive traits in this species or the role of root symbioses in tolerance to metals, are suggested.

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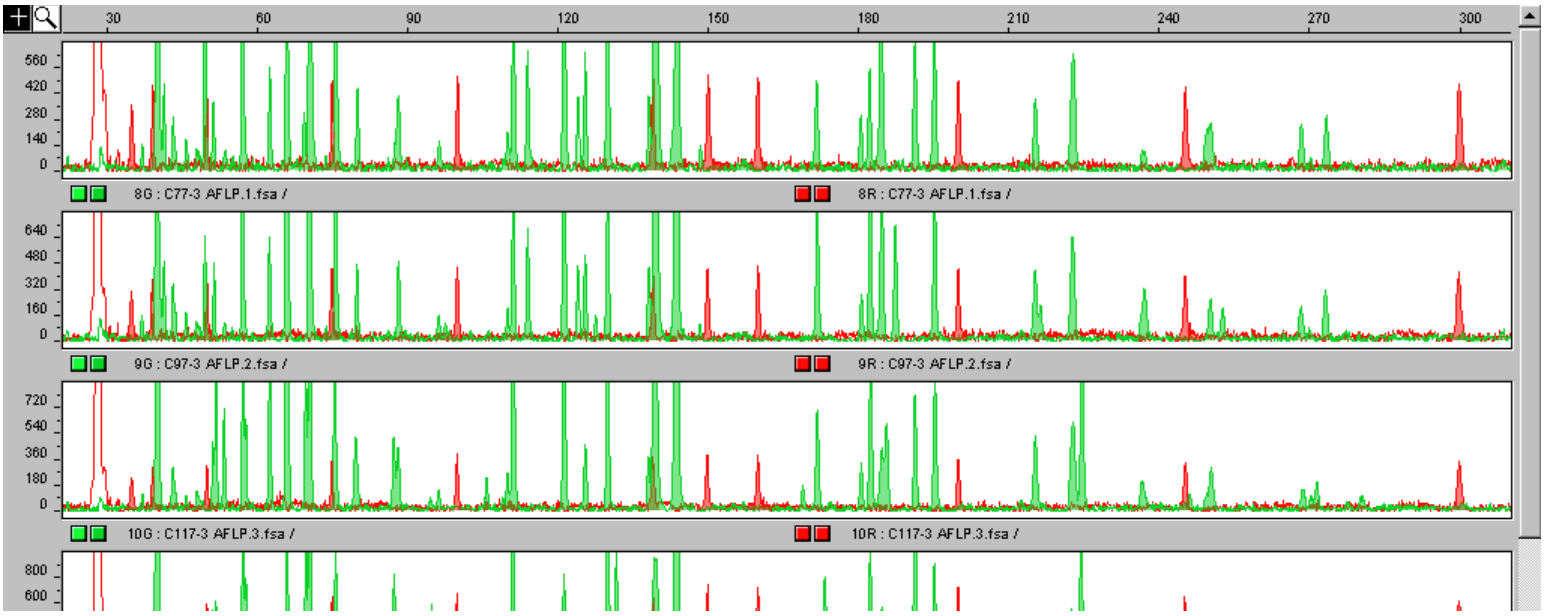
Chapter 6

AFLP analysis of the pseudometallophyte *Cistus ladanifer*: a comparison with cpSSRs and an exploratory genome scan to investigate loci associated to soil variables



Previous page: Screenshot of electropherograms of different *C. ladanifer* plants genotyped with AFLP markers. Red peaks correspond to the molecular weight marker, whereas green peaks indicate loci amplified with the primer pair: NED-EcoRI- AGG/ MseI- CAG. (Image: C. Quintela-Sabarís)

CHAPTER 6

AFLP analysis of the pseudometallophyte *Cistus ladanifer*: a comparison with cpSSRs and an exploratory genome scan to investigate loci associated to soil variables

Keywords: Amplified Fragment Length Polymorphism, chloroplast microsatellites, *Cistus ladanifer*, Generalized Estimating Equations, heavy metals

ABSTRACT

Cistus ladanifer is a pseudometallophyte which grows in different metal-rich substrates from the Western Mediterranean area.

We have analysed 33 populations of this species with AFLPs in order to i) assess the genetic patterns of colonisation of metalliferous areas by *C. ladanifer* obtained with an AFLP genome scan and to compare them with previous cpSSRs results and ii) identify loci potentially linked to tolerance to metalliferous soils.

AFLP results were partially congruent with previous cpSSR results, revealing no influence of soil type on the genetic diversity and differentiation of this species. However, some differences arose, mainly due to different properties (mutation rate, ploidy level, inheritance, …) inherent in each marker.

Then we used Generalized Estimating Equations models to test the correlation between allele distribution and soil data. These regression analyses showed that the total soil contents of Mn has an important effect on allele distribution in *Cistus ladanifer*, the strongest in relation to the other soil variables we also have analysed.

Moreover, we report the detection of a particular allele with a possible role in tolerance to high Mn concentrations in soils.

6.1 Introduction

Soils with high concentrations of metals (metalliferous soils) are toxic to most plants and other living organisms (Shaw *et al.* 2004). Moreover, soils polluted by human activities, particularly those activities related to the production of metals (mine tailings, smelting areas ...) constitute a threat to the environment and public health: they usually have a sparse (or even absent) plant cover and thus metals can leach into groundwater or the polluted soil can be dispersed by wind which then affects productive agricultural land or natural reserves (Ruttens *et al.* 2006, Tordoff *et al.* 2000). However, the metalliferous areas also may be considered as ecological islands in which different metal-tolerant plant species are found. Thus, soils with high contents of heavy metals provide the opportunity to study the establishment and differentiation of plant populations under severe selection pressure (Lefèbvre and Vernet 1990).

Among metal-tolerant species we may distinguish between strict metallophytes (or eumetallophytes), whose populations only grow on metalliferous substrates, and pseudometallophytes (or facultative metallophytes), which can grow both in metalliferous and non-metal-

Table 6.1: Description of *Cistus ladanifer* populations studied.

Pop (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat	N
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with scattered *Pinus pinea* and *P. halepensis*	4.79º W	36.84º N	7
Almodóvar (EAL)	North	M (h)	Clays close to a road	Grassland with dispersed *C. ladanifer* plants	5.65º W	36.16º N	8
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N	8
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N	9
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N	8
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N	9
Despeñaperros (EDE)	North	M (h)	Quartzites, close to the highway.	Dense *Cistus ladanifer* shrubland with *Quercus ilex* and *Juniperus oxycedrus*	3.51º W	38.39º N	8
Fuente Saúco (EFS)	North	NM	Sandstone and conglomerates	Shrubland with *Quercus ilex*	5.51º W	41.19º N	7
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N	8
Grazalema (EGR)	South	NM	Decarbonated limestone	Shrubland with *C. ladanifer* and *C. monspelliensis*	5.27º W	36.78º N	9
Guadarrama (EGU)	North	NM	Granite	Dense shrubland with isolated *Quercus ilex*	4.10º W	40.68º N	9
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N	9
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N	8
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N	5
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N	9
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N	8
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N	9
Valdecaballeros (EVC)	North	NM	Sedimentary material (gravels, clays)	Dense shrubland with *Pinus pinaster*	5.34º W	39.33º N	7
Bni Hadifa (MBH)	South	NM	Sandstones	Open *Pinus halepensis* forest	4.17º W	35.02º N	8
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N	7
El Jebha (MEJ)	South	NM	Sandstone	Open *Pinus halepensis* forest	4.64º W	35.18º N	9
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks and *Cistus laurifolius*	4.64º W	34.95º N	7
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N	8

Lineage and Type were defined on the basis of analyses published in Quintela-Sabarís *et al*. 2010. *Lineage:* cpSSR lineage ('North', 'South'); *Type*: M (metallicolous), NM (non-metallicolous). Geographic coordinates are given in decimal degrees. *N*: number of plants genotyped with AFLP in each population.

Table 6.1: (continued)

Pop (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat	N
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89° W	35.29° N	7
Tanger (MTA)	South	NM	Sandstone	Open *Pinus pinaster* forest	5.93° W	35.78° N	8
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18° W	37.88° N	7
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87° W	41.85° N	8
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78° W	37.07° N	8
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03° W	37.39° N	6
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82° W	41.52° N	6
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75° W	41.78° N	7
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98° W	37.03° N	7
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29° W	40.43° N	9

Lineage and Type were defined on the basis of analyses published in Quintela-Sabarís *et al.* 2010. *Lineage*: cpSSR lineage ('North', 'South'); *Type*: M (metallicolous), NM (non-metallicolous). Geographic coordinates are given in decimal degrees. *N*: number of plants genotyped with AFLP in each population.

liferous soils (Pollard *et al.* 2002).

Due to the aforementioned duality of metalliferous areas, pseudometallophytes are interesting organisms since i) they constitute highly relevant models to study local adaptation in plants (Linhart and Grant 1996); and ii) they usually possess, along with metal tolerance, several traits (high adaptability to adverse soil conditions, high biomass production, good competitiveness…) that may make them useful for phytoremediation technologies (Poschenrieder *et al.* 2001).

The population genetics of different pseudometallophyte species has been investigated in order to identify evolutionary and genetic factors involved in tolerance (Westerbergh and Saura 1992, Vekemans and Lefèbvre 1997, Mengoni *et al.* 2001, Jiménez-Ambriz *et al.* 2007, Pauwels *et al.* 2008).

These studies mainly tried to identify whether metallicolous populations suffered a founder effect during the colonisation of metalliferous areas or to determine if metallicolous populations of a particular species share a common ancestry or whether they are the result of local colonization events. Their results show that metallicolous populations are usually the result of local evolution. However, regarding the occurrence of founder effect, a common trend across species was not found. Pauwels *et al.* (2005) proposed that

the colonisation of metal-polluted environments is associated with a genetic bottleneck in species with populational tolerance (e.g. *Silene paradoxa*, Mengoni *et al.* 2001), whereas in species with constitutive (or 'specieswide') tolerance (such as *Arabidopsis halleri*, Pauwels *et al.* 2005) the effect of a bottleneck may not be detected.

Among the molecular markers applied to non-model pseudometallophytes, those based on maternally-inherited chloroplast DNA (such as chloroplast microsatellites- cpSSR), are useful to infer the phylogeography of a species, making possible a better understanding of the effect of metal pollution on the genetic structure of its populations (Staton *et al.* 2001). However, chloroplast markers are mainly neutral and seldom related to metal tolerance.

In contrast, AFLP markers (Vos *et al.* 1995), which have predominantly a nuclear origin (Meudt and Clarke 2007) can be applied to any organism without previous knowledge of sequences. Thus, this kind of marker has allowed genome scans to be applied to non-model organisms (Bonin *et al.* 2007). The use of genome scans along with environmental data allows the detection of those markers potentially linked to adaptive loci (Holderegger *et al.* 2008) among many markers (such as AFLP). Thus, AFLP loci with potential ecological relevance have been identified in *Arabis alpina* (Poncet *et al.* 2010), or, interestingly, Meyer *et al.* (2009) have inferred loci putatively involved in tolerance to heavy metals in metallicolous (M) and non-metallicolous (NM) populations of the pseudometallophyte *Arabidopsis halleri*.

Cistus ladanifer L. (Cistaceae) is a pseudometallophyte shrub native to the Western Mediterranean region (from S of France to the N of Morocco and Algeria, Demoly and Montserrat 1993). In addition to its adaptability to disturbances occurring in Mediterranean areas, mainly fires (Pérez-García 1997) and water and light stress (Martín Bolaños and Guinea López 1949, Núñez-Olivera *et al.* 1996), this species has been described as interesting for phytostabilisation procedures and also for phytoextraction of Zn in soils with low to medium contents of this metal (Díez-Lázaro 2008).

In a previous paper we analysed 33 NM and M (serpentine and mines) *Cistus ladanifer* populations with cpSSR markers, from almost its entire distribution area (Quintela-Sabarís *et al.* 2010). We inferred that M populations evolved in parallel within two independent chloroplast lineages.

In this work, we re-analyse the same 33 populations with AFLP markers with two main aims: i) to assess and to compare the genetic structure and the patterns of colonisation of metalliferous areas by *C. ladanifer* obtained using AFLPs (nuclear markers, dispersed by pollen and seeds) and cpSSRs (maternally-inherited, and thus dispersed only through seeds (Guzmán and Vargas 2009)) analyses, and ii) to identify AFLP loci potentially associated to tolerance to metalliferous soils in this species.

We addressed the latter topic through the analysis of correlation be-

Table 6.2: Summary of amplification results and range of fragment sizes finally used in the analyses. Error rates were computed as indicated in Bonin *et al.* (2004). The correlation size/frequency is an indication of possible size homoplasy (Vekemans *et al.* 2002).

		Range of fragment sizes		
		50-500 bp	**100-500 bp**	**150-500 bp**
N of fragments	Combination A	64	57	50
	Combination B	80	73	60
	Total	**144**	**130**	**110**
Error rate	Combination A	0.074	0.069	0.064
	Combination B	0.054	0.055	0.048
	Total	**0.063**	**0.061**	**0.055**
Correlation Size/Frequency	Pearson index	-0.2653	-0.2168	-0.1292
	P	**0.001**	**0.013**	**0.178**

P: probability of obtaining a more extreme correlation value than that presented by chance alone.
Combination A: FAM-EcoRI- ACT/ MseI- CTG; ***Combination B***: NED-EcoRI- AGG/ MseI- CAG

tween the distribution of AFLP markers and total metal contents in soils and applying generalized estimating equations (GEE) to correct for phylogeographic autocorrelations of individuals within chloroplast lineages.

6.2 Material and Methods

6.2.1 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire natural geographic range of this species were sampled. The subspecies growing in each site were identified on the basis of morphological traits. We included M populations from different geographic areas: ultramafic outcrops of Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), M populations growing on mine tailings and in the vicinity of highways from the Centre and South of the Iberian Peninsula and non-metallicolous (NM) populations (Table 6.1).

To collect the plant material a longitudinal transect was established for each population. Ten plants, separated by at least 5 m, were selected along each transect, and ripen fruits were collected from them. The seeds were sown and seedlings grown in the laboratory. One seedling per mother plant was selected for subsequent analyses. Young plants were frozen in liquid nitrogen and kept at -20ºC until DNA extraction.

In addition, in each site one (or two) soil samples were collected from 5 to 15 cm in depth. Each soil sample was air-dried and sieved through a 2 mm-mesh.

6.2.2 Soil analysis and variables

We initially considered nineteen soil variables for our statistical analyses: soil pH,

ratio of total Ca:Mg, ratio of extractable Ca:Mg, total content of As and Sb, and total and extractable contents of Co, Cr, Cu, Mn, Ni, Pb, and Zn (See Tables S 3.1 and S6.1 in Supplementary Material).

Total concentrations of Ca and the metalloid As were quantified in solid soil subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF), whereas other subsamples were digested with HNO_3 for the quantification of total Mg contents with Inductively Coupled Plasma -Optical Emission Spectrometry (ICP-OES) and for the quantification of total Sb with ICP-Mass Spectrometry (ICP-MS). Extractable Ca and Mg were quantified with Atomic Absorption Spectrometry in liquid soil extracts obtained following Quintela-Sabarís *et al.* (2010). Data of pH and total and extractable contents of the other metals were previously published (Quintela-Sabarís *et al.* 2010).

We performed a Principal Component Analysis on these variables in order to check the correlation among them. We finally retained only the most uncorrelated variables from the PCA: soil pH, Ca:Mg ratio and total soil contents of Mn, Ni, Pb, Sb and Zn. These seven variables were then used as explanatory soil variables in the identification of loci under directional selection.

6.2.3 DNA extraction

The DNA was extracted from 100 mg of frozen leaves using Dneasy® Plant Mini Kit (QIAGen), following the manufacturer's indications. In some cases an additional wash with 500 µl of absolute ethanol was needed in order to remove secondary compounds from the DNA extracts.

6.2.4 AFLP protocol and data scoring

To optimize the AFLP conditions and to select the primer-enzyme combinations to use, a preliminary study with 30 individuals from geographically separated populations and 64 combinations of selective primers was carried out using AFLP Plant Mapping Kit (Applied Biosystems). After the optimization procedure, we selected two combinations of selective primers that yielded reliable polymorphic banding patterns: **combination A** (EcoRI- ACT/MseI-CTG) and **combination B** (EcoRI- AGG/MseI- CAG). In addition, we introduced the following modifications in the manufacturer's protocol: (1) each restriction-ligation reaction was diluted with 11.0 µl of $TE_{0.1}$ buffer rather than 189.0 µl, (2) pre-selective amplification products were diluted with 40.0 µl $TE_{0.1}$ buffer instead of 190.0 µl, (3) each selective amplification was performed in a final volume of 12.5 µl instead of 25 µl.

Fluorescent labelled selective PCR products were separated using an ABI 3130 xl (Applied Biosystems) DNA

Table 6.3: Measures of intrapopulation genetic diversity. **PPL**: percentage of polymorphic loci. H_j: Nei's genetic diversity (and its standard deviation); **SMC**: Simple Matching Coefficient (and its standard deviation). In addition, we present data of genetic diversity computed on the basis of cpSSR in the same populations included in this study (Quintela-Sabarís *et al.* 2010).

Pop	Type	AFLP allele-based measures			AFLP band-based measure		cpSSR measures (Quintela-Sabarís et al. 2010)		
		PPL	H_j	S.D.(H_j)	1-SMC	S.D. (1-SMC)	H_E	$r_{(7)}$	D^2_{SH}
EAC	M	22.7	0.06	0.01	0.07	0.03	0.00	0.00	0.00
EAL	M	32.7	0.11	0.02	0.12	0.04	0.00	0.00	0.00
EBE	NM	36.4	0.11	0.02	0.12	0.05	0.64	2.33	0.76
ECA	NM	31.8	0.09	0.01	0.08	0.03	0.71	1.93	0.89
ECO	M	33.6	0.10	0.01	0.10	0.03	0.56	1.00	0.28
ECR	NM	30.9	0.08	0.01	0.08	0.03	0.36	0.93	0.18
EDE	M	34.5	0.11	0.02	0.10	0.04	0.64	1.70	0.46
EFS	NM	33.6	0.11	0.02	0.09	0.03	0.64	1.70	0.46
EGJ	NM	36.4	0.12	0.02	0.10	0.03	0.47	0.99	0.23
EGR	NM	31.8	0.08	0.01	0.08	0.03	0.00	0.00	0.00
EGU	NM	37.3	0.09	0.01	0.08	0.03	0.20	0.70	0.10
EMA	NM	30.0	0.08	0.01	0.06	0.02	0.62	1.87	1.78
EMF	NM	26.4	0.07	0.01	0.06	0.02	0.00	0.00	0.00
ERB	NM	30.0	0.10	0.01	0.07	0.02	0.53	1.00	0.27
ESB	M	38.2	0.11	0.02	0.10	0.06	0.00	0.00	0.00
ESP	M	27.3	0.08	0.01	0.07	0.03	0.42	1.56	0.50
ETO	M	26.4	0.07	0.01	0.04	0.03	0.20	0.70	0.10
EVC	NM	30.0	0.09	0.01	0.07	0.02	0.71	2.39	0.71
MBH	NM	31.8	0.10	0.01	0.07	0.04	0.20	0.70	0.10
MBT	NM	31.8	0.10	0.01	0.08	0.03	0.20	0.70	0.10
MEJ	NM	24.5	0.07	0.01	0.06	0.04	0.00	0.00	0.00
MKE	NM	25.5	0.08	0.01	0.06	0.03	0.38	1.40	0.22
MSI	M	22.7	0.07	0.01	0.05	0.02	0.53	1.00	0.27
MSII	M	30.0	0.09	0.01	0.08	0.04	0.70	2.16	0.58
MTA	NM	31.8	0.09	0.01	0.08	0.02	0.00	0.00	0.00
PAL	M	28.2	0.09	0.01	0.08	0.03	0.64	1.78	0.53
PBR	M	41.8	0.14	0.02	0.14	0.06	0.53	1.00	0.27
PBU	NM	40.0	0.13	0.02	0.13	0.06	0.39	0.97	0.19
PCF	NM	47.3	0.15	0.02	0.17	0.07	0.39	0.97	0.19
PMC	M	31.8	0.10	0.01	0.09	0.04	0.00	0.00	0.00
PSA	M	37.3	0.12	0.02	0.11	0.05	0.00	0.00	0.00
PSV	NM	42.7	0.15	0.02	0.15	0.05	0.20	0.70	0.10
PVE	NM	42.7	0.13	0.02	0.11	0.03	0.00	0.00	0.00
Mean (±S.D.)		32.7 ± 6.0	0.098 ± 0.024		0.090 ± 0.030		0.32 ± 0.27	0.92 ± 0.77	0.28 ± 0.37

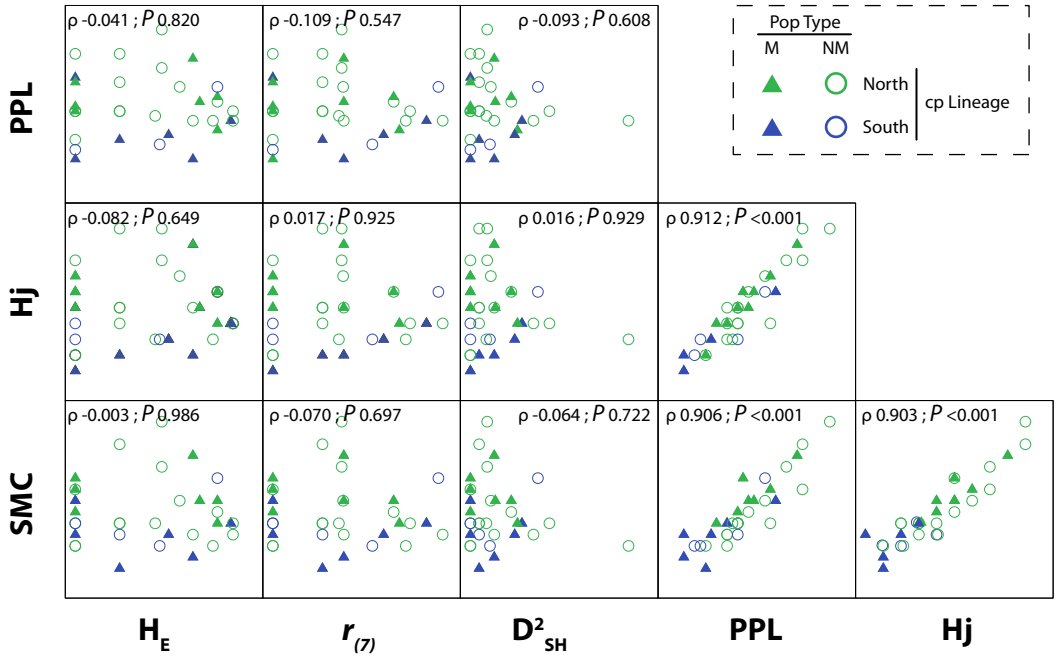


Figure 6.1: Relationships among different AFLP-based estimates of intrapopulation genetic diversity and others estimates of genetic diversity based on cpSSR markers (Quintela-Sabarís *et al.* 2010). Different symbols indicate population type (M and NM) and chloroplast lineage (North or South). Based on AFLP: **PPL**, % of polymorphic loci; **Hj**, Nei's gene diversity; **SMC**, 1-(simple matching coefficient). Based on cpSSR: $\mathbf{H_E}$, haplotypic diversity (Nei 1987); $\boldsymbol{r_{(7)}}$, haplotype richness after rarefaction to a population size of 7 plants (El Mousadik and Petit 1996); $\boldsymbol{D^2_{SH}}$ measure (Vendramin *et al.* 1998). The value of Spearman's $\boldsymbol{\rho}$ ('rho') correlation index and ***P*** (probability of obtaining a more extreme ρ value than that presented by chance alone) are presented in each graph.

analyser. The ABI files with electropherograms were visualized and subsequently scored using the open source program Genographer v 2.0 (Benham 2001, modified by Travis Banks and available at http://sourceforge.net/projects/genographer/). Genographer converts the electropherograms into a gel-like image which is easier to score by visual screening. Afterwards, we applied a correction, which eliminates many of the slight deviations that exist from lane to lane, and resulted effective in fixing compressions or expansions along the length to the lane.

The AFLP markers have 2 alleles, so were scored as band presence (1) or band absence (0). Those plants showing weak or very awkward profiles were removed from the analysis. The quality of PCR amplifications and bin scoring was assessed as following: 20 plants (6.5% of plants analysed) were re-extracted and amplified independently. An error rate was computed as the sum of errors/the total number of comparisons (Bonin *et al.* 2004). Those bins with an error rate equal or higher than 0.2 were removed from the analysis.

Table 6.4: Results of the non-parametrical Mann-Whitney tests on the intrapopulation genetic diversity data. Comparisons between M (metallicolous) and NM (non-metallicolous) populations were made for the whole set of populations and separately for each chloroplast lineage. For each estimator of genetic diversity (**PPL**: % of polymorphic loci. H_j: Nei's gene diversity. **SMC**: 1-Simple Matching Coefficient), we present: ***U***, Mann-Whitney statistic. m_{NM} and m_M refer to the median value of the estimator for the groups of Non-Metallicolous (NM) and for Metallicolous (M) populations, respectively. n_{NM} and n_M refer to the number of populations of NM and M populations within each group. ***P***: probability of obtaining a more extreme *U* value than that presented by chance alone.

Div Estimator	Data set	*U*	m_{NM}	n_{NM}	m_M	n_M	*P* (2-tailed)
PPL	Cp lineage 'North'	48.0	32.70	14	33.60	7	0.940
	Cp lineage 'South'	12.0	31.80	6	26.85	6	0.332
	All populations	108.5	31.80	20	31.80	13	0.426
H_j	Cp lineage 'North'	41.0	0.10	14	0.11	7	0.547
	Cp lineage 'South'	12.0	0.09	6	0.08	6	0.328
	All populations	120.5	0.10	20	0.10	13	0.724
SMC	Cp lineage 'North'	31.0	0.08	14	0.10	7	0.175
	Cp lineage 'South'	14.0	0.08	6	0.07	6	0.515
	All populations	122.0	0.09	20	0.08	13	0.766

Depending on the selective primer combinations, markers were named A or B, followed by a number indicating its fragment size (in bp). Initially, all fragments between 50 and 500 bp were considered, but, as posterior analyses revealed size homoplasy in the lowest bp ranges (as previously found by Vekemans *et al.* 2002; see following section), only the fragments from 150 bp to 500 bp sizes were considered.

6.2.5 Data analysis: within population diversity and genetic structure of populations

The genetic diversity within population estimates were computed based on two different approaches: the allele frequency-based metrics (% of polymorphic loci, **PPL**; and the Nei's gene diversity per locus, H_j, after Lynch and Milligan (1994)) and the band-based metric (Simple Matching Coefficient, **SMC**).

Allele frequency-based metrics were computed using AFLP-SURV 1.0 software (Vekemans 2002). To estimate allele-frequencies, we used a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), and assumed a Hardy-Weinberg genotypic proportions ($F_{IS} = 0$). This is a reasonable assumption, since *Cistus ladanifer* is an obligatory-outcrossing species, with a gametophytic mechanism of incompatibility (Talavera *et al.* 1993), and in allogamous species allele frequencies usually do not violate the Hardy-Weinberg Equilibrium (Meudt and Clarke 2007).

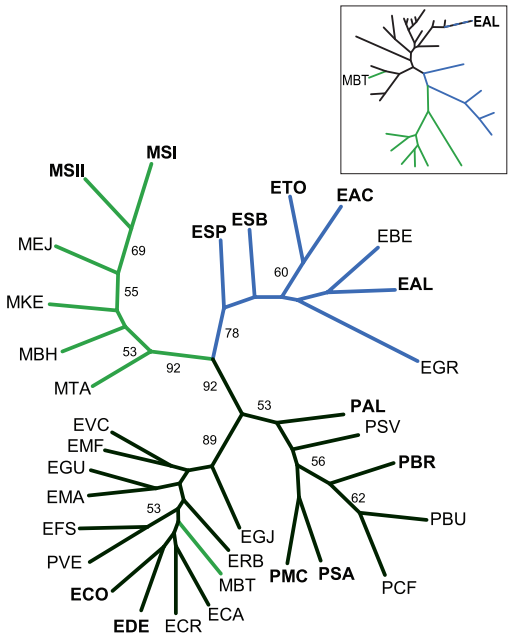


Figure 6.2: Unrooted consensus dendrogram based on 1000 bootstrapped trees. Population codes are the same as table 6.1. Nei's D distance between populations (after Lynch and Milligan 1994) was used. Numbers indicate the percentage of support for each branch of the dendrogram. Only bootstrap values higher than 50 are reported. A scheme of the consensus dendrogram obtained previously with cpSSR on the same populations (Quintela-Sabarís *et al.* 2010) is presented in the upper right corner of the figure. Green lines indicate the position of populations from the North of Morocco, whereas blue lines indicate populations from the Betic area (South and South-east of the Iberian Peninsula). M populations are indicated by **bold type**.

We computed the Pearson correlation coefficient between fragment size and fragment frequencies on the overall sample with the same software (Vekemans *et al.* 2002). After preliminary analyses with different size ranges, non significant values of Pearson coefficient were obtained using fragments in the range 150-500 bp (Table 6.2), so we selected this range to perform the statistical analyses.

The Simple Matching Coefficient (SMC) was computed within each population using the SIMQUAL module from NTSYS-pc software (v. 2.11L; Rohlf, 2002). The SMC, which takes into account both shared 1's (presence of a band) and shared 0's (absence of a band), was proposed as the best metric for comparisons of phenotypic similarity with dominant markers within a single diploid species (Kosman and Leonard, 2005). SMC is a measure of similarity, therefore we computed the dissimilarity for each population as: (1-SMC).

Differences in the levels of intrapopulation diversity between M and NM populations were assessed using the nonparametrical Mann-Whitney test, which is equivalent to Student's t-test, but without the assumption of the normality of data and a better robustness against outliers. Separate analyses were performed on the whole set of 33 populations and separately for each chloroplast lineage, using PPL, Hj and SMC data.

The estimates of intrapopulation genetic diversity obtained with AFLP markers were compared with those obtained previously with cpSSR (Quintela-Sabarís *et al.* 2010) using the Spearman correlation index, which does not request data normality. The Mann-Whitney tests and the Spearman correlation indices were performed with the SPSS package (v. 15, SPSS Inc., Chicago, IL, USA).

A pairwise matrix of genetic distances between populations was com-

puted based on Nei's genetic distance, D (after Lynch and Milligan 1994), using the AFLP-SURV. The genetic distances were validated by a bootstrap procedure with 1000 replications. Each bootstrapped matrix was used to compute a Neighbour-Joining (NJ, Saitou and Nei 1987) dendrogram with the program NEIGHBOR. The program CONSENSE allowed us to construct a consensus dendrogram on the basis of 1000 NJ dendrograms. NEIGHBOR and CONSENSE belong to the package PHYLIP (v. 3.6, Felsenstein 2004).

We computed the correlation between pairwise D matrix (obtained with AFLP) with (a) the matrix of genetic distance obtained with cpSSR markers (based on Cavalli-Sforza and Edwards distances, Quintela-Sabarís *et al.* 2010), and with (b) a matrix of pairwise geographic distances (expressed as natural logarithm of Km, Ln(km)) using the Mantel matrix-correspondence test (Mantel 1967), which is included in the module MXCOMP from NTSYS-pc (v. 2.11L; Rohlf, 2002). We set 10000 permutations to validate the results.

In order to infer population genetic structure, Bayesian analysis using a spatial clustering model implemented in BAPS software version 5.4 was performed (Corander *et al.* 2008). The spatial clustering of groups model was run using each population, with known coordinates, as the unit to be clustered. We initially fixed *k* (the number of clusters) from 2 to 33. Afterwards, we selected the value of *k* that had the minimum log marginal likelihood and repeated the analysis 100 times to obtain the optimal population structure. The use of spatial information increases the power to correctly detect the underlying population structure (Bonin *et al.* 2007).

The results of the mixture analysis were then used to perform an admixture analysis, following the protocol by Corander and Marttinen (2006). We used the following settings: (1) minimal size of clusters. five individuals; (2) 200 iterations to estimate the admixture coefficients for the individuals; (3) 300 simulated reference individuals from each population, and (4) 20 iterations to estimate the admixture coefficients for the reference individuals. According to Corander and Marttinen (2006) BAPS performs equally well or even better than the widely used program STRUCTURE (Pritchard *et al.* 2000) with a 400-fold speed advantage.

The population genetic structure was further explored using a locus-by locus Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992), implemented with the Arlequin 3.5 software (Excoffier *et al.* 2005). This test allowed for estimation of variance components between individuals within populations, between populations within groups and among groups. The groups were defined either on the basis of soil analyses or on the basis of BAPS-clustering. Variance components and Φ statistics were estimated for each locus and then combined to produce synthetic estimators of Φ statistics. The significance values were computed by a permutation test from 20000 permuted matrices.

Finally, we computed the pollen-to-seed migration ratio($r = m_p/m_s$) follow-

ing Petit *et al.* (2005). We estimated the genetic subdivision at nuclear markers (Φ_{STb}) using Arlequin 3.5 software (Excoffier *et al.* 2005), whereas the subdivision at maternally inherited markers (Φ_{STm}) was obtained from a previous study (Quintela-Sabarís *et al.* 2010).

6.2.6 Data analysis: detection of ecologically relevant loci using GEE

We used generalized estimating equations (GEE) in order to detect alleles that were correlated to soil variables. GEE are an extension of generalized linear models (Carl and Kuhn 2007), which may consider autocorrelation between samples by including an additional variance component directly into the independent data model's estimating equation to accommodate correlated data. This method allows us to consider that neighbouring individuals within chloroplast lineages are genetically more similar than individuals belonging to different chloroplast lineages which, as we inferred in a previous work (Quintela-Sabarís *et al.* 2010), may be isolated from each other from the Last Glacial Maximum at least. As we dealt with binary data, we used a logit-link and binomial error distribution to correlate allele occurrence for each AFLP locus per sampling location to quadratic polynomials of environmental variables. To consider the variety of response curve shapes other than a linear response (Legendre and Legendre 1998) we used quadratic polynomials. In order to select the best GEE models, we used the quasi-likelihood information criterion (QIC) adapted by Pan (2001). The best model is that with the lowest QIC. All combinations of variables were investigated for each locus. Finally, we tested 128 GEE (from the null model to the full model with seven variables in second-order polynomials) on our data set. All GEE models were calculated using the R package *geepack* (Yan and Fine 2004), and an implementation developed by Poncet *et al.* (2010) for the QIC calculation in R (R Development Core Team 2007). For each selected model, we also tested each regression coefficient using the Wald test. To avoid a high rate of false positives due to multiple tests, significance levels were calculated for each regional data set by minimizing the false discovery rate (FDR; Storey 2002). *P*-value rejection thresholds were adjusted so as to have less than one false positive locus per data set and were estimated using the R package *qvalue*.

6.3 Results

A total of 109 and 112 different fragments were amplified with primer combinations A and B, respectively. However, after the filtering process (error rates and size ranges) 110 markers were finally considered (50 obtained with combination A and 60 with combination B). The total error rate using both combinations was 0.055 (Table 6.2).

6.3.1 Genetic diversity and differentiation

The values of genetic diversity within populations are presented in Table 6.3. The Mann-Whitney tests did not reveal significant differences in diversity between M

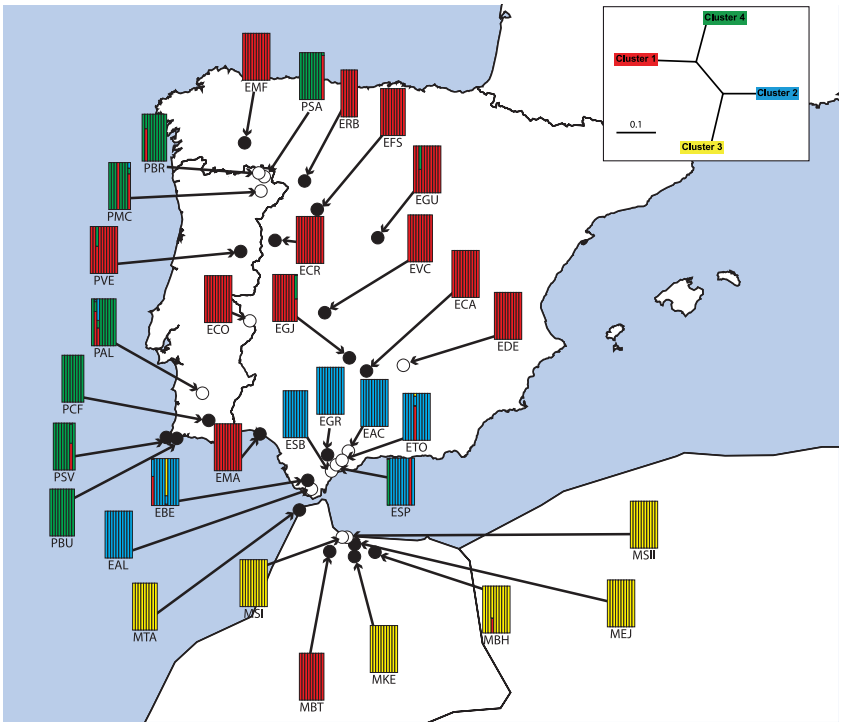


Figure 6.3: Localities for the 33 sampled populations of *Cistus ladanifer* (for details of each population see Table 6.1). Black circles indicate NM populations whereas white circles indicate M populations. The graphs next to each population indicate the proportional assignment of individuals from each population to the four different clusters as detected in an admixture analysis of AFLP data conducted with the program Bayesian Analysis of Population Structure (BAPS). Every vertical coloured bar corresponds to an individual. A vertical bar is split into several colours when there is evidence for admixture. The BAPS-defined clusters are: cluster 1 (red), cluster 2 (blue), cluster 3 (yellow) and cluster 4 (green). Only significant admixtures (significance level 5%) are showed. A NJ dendrogram showing the relationships among each of those clusters is presented on the upper right corner. NJ dendrogram is based on Kullback-Leibler distances among clusters.

and NM populations, neither when considering the whole set of populations nor each chloroplast lineage separately (Table 6.4).

We found high and significant linear correlations among the three diversity estimates computed on AFLP data. Interestingly, the estimates of genetic diversity based on AFLP and cpSSR were not correlated (Figure 6.1).

The matrix of Nei's D genetic distances among populations is available in Supplementary Material S 6.2. As previously obtained with chloroplast markers, the consensus dendrogram revealed a clustering more related to phylogeography than to soil type (Fig. 6.2). A group of populations of *C. ladanifer* subsp. *africanus* from the North of Morocco is defined (marked in green colour in Fig.

Table 6.5: Summary of analysis of molecular variance (AMOVA) of *Cistus ladanifer* (a) considering the whole data set, (b) be tween population types (M vs. NM), (c) between BAPS-defined groups (*K* = 4) and (d to g) separate analyses between population types for each of the four groups defined by SAMOVA analysis. *SS* = sum of squared deviation, ***P*** = level of probability of obtaining a more extreme component estimate by chance alone. n.s. = not significant (5% level).

Analysis	Source of variation	SS	Variance components	% Total Variance	*P*
(a) Whole data set (Φ_{ST}= 0.35)	Among populations	836.74	2.62	35.47	< 0.0001
	Within populations	1126.61	4.77	64.53	
	Total	1963.35	7.39		
(b) M vs NM	Between population types (Φ_{CT} = 0.01)	40.87	0.11	1.52	0.02
	Among pops. within population types	795.87	2.57	34.44	< 0.0001
	Within populations	1126.61	4.77	64.04	< 0.0001
	Total	1963.35	7.45		
(c) BAPS mixture results	Among BAPS-defined clusters (Φ_{CT} = 0.31)	515.69	2.50	31.04	< 0.0001
	Among pops. within clusters	321.05	0.77	9.62	< 0.0001
	Within populations	1126.61	4.77	59.33	< 0.0001
	Total	1963.35	8.04		
(d) Cluster 1	Between population types (Φ_{CT} = 0.01)	12.68	0.06	1.06	n.s.
	Among pops. within population types	117.23	0.74	13.72	< 0.0001
	Within populations	435.09	4.57	85.22	< 0.0001
	Total	565.00	5.37		
(e) Cluster 2	Between population types (Φ_{CT} = -0.03)	9.97	-0.14	-2.84	n.s.
	Among pops. within population types	66.39	1.01	20.12	< 0.0001
	Within populations	229.43	4.16	82.71	< 0.0001
	Total	305.79	5.03		
(f) Cluster 3	Between population types (Φ_{CT} = 0.002)	13.03	0.01	0.16	n.s.
	Among pops. within population types	52.45	1.11	22.63	< 0.0001
	Within populations	159.39	3.78	77.21	< 0.0001
	Total	224.87	4.90		
(g) Cluster 4	Between population types (Φ_{CT} = 0.01)	9.77	0.07	1.00	n.s.
	Among pops. within population types	39.52	0.15	2.19	0.04
	Within populations	302.70	6.78	96.81	0.01
	Total	351.99	7.01		

6.2). In addition, another cluster groups all the populations from South and SE of the Iberian Peninsula (Betic Area, marked in blue in Fig. 6.2). Populations from the rest of Iberian Peninsula are arranged in two other clusters. Population MBT from the North of Morocco, which belongs to *C. ladanifer* subsp. *ladanifer* is located next to populations of the same subspecies from Iberian Peninsula. Each of the four defined clusters includes both M and NM populations.

The congruency between AFLP and chloroplast markers is also supported by the fact that a Mantel test (1967) revealed that genetic distances among populations based on AFLP or on cpSSR were positively correlated (the value of correlation factor r was 0.5688; *P* 0.0001). However, the population EAL showed differences between AFLP and cpSSR: on the basis of AFLP it is placed in a cluster with other populations from the Betic area, but if we consider cpSSR this population is more linked to populations from SW to the North of Iberian Peninsula (Fig. 6.2). Mantel test revealed a low but significant correlation between matrices of genetic and geographic distances between populations (r = 0.3923; $P < 0.0001$).

Bayesian mixture analysis inferred four clusters of populations (k = 4; Log(marginal likelihood) of optimal partition: -6 099.8331) which fit the four clusters identified in NJ consensus dendrogram (Fig. 6.3). The degree of admixture among clusters is low, and it occurs mainly in populations from the Iberian Peninsula. It is remarkable the fact that no individual plant from population MBT (*C. ladanifer* subsp. *ladanifer* from N of Morocco) showed indications of admixture with the north African cluster 3 (marked in yellow). In contrast population EBE (*C. ladanifer* subsp. *africanus* from S of Iberian Peninsula) presents an indication of admixture with the cluster 3. A with the consensus dendrogram, each of the four defined clusters includes both M and NM populations (Fig. 6.3).

The AMOVA analysis inferred that most of the molecular variance occurs at the intra population level (64.53%; Table 6.5a), and a Φ_{STb} value of 0.35 ($P < 0.0001$). AMOVA also revealed a significant partitioning of 31.04% of molecular variance among the four clusters defined by BAPS ($\Phi_{CT} = 0.31$; $P < 0.0001$; Table 6.5c). In contrast, the comparisons between population types at the global level (Table 6.5b) revealed that less than 2% of molecular variance occurs between M and NM populations. The differentiation between population types became non-significant when the AMOVA analyses where performed within each BAPS cluster (Table 6.5d to 6.5g).

6.3.2 Detection of relevant loci using GEE

GEE were used to correlate allele frequencies to soil variables correcting for chloroplast lineages. The threshold for significance minimizing the false discovery rate was estimated as 0.0031. Sixteen loci (14.5% of total loci) were significantly correlated to one or two soil variables (Fig. 6.4), giving a total of 21 significant

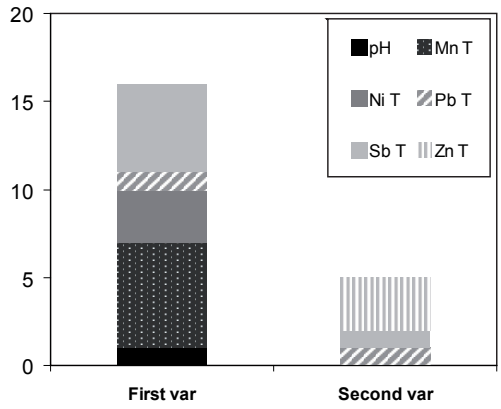


Figure 6.4: Number of AFLP loci significantly correlated with soil variables in the generalized estimating equations (GEE) applying a correlation for chloroplast lineage. Sixteen bands (14.5% of obtained loci) were correlated with one variable, 5 of which were also correlated with a second variable. Different fill patterns represent pH and Total soil contents of Mn, Ni, Pb, Sb and Zn.

relationships (Supplementary Material S 6.3). MnT and SbT were the soil variables with most influence, since each of them represented 28.6% of detected significant correlations. NiT and ZnT accounted for 14% each. No locus was significantly related to Ca:Mg ratio.

In order to prune possible false positives, we further explored these 21 relationships by i) performing separate Mann-Whitney U tests to detect significant differences in soil variables between the group of plants where a certain band was absent or present, and ii) observing the geographic distribution of the presences/absences of each locus.

The Mann-Whitney U tests showed significant differences for only 8 loci (5 of them related to MnT), whereas in the case of the loci A492 and B346 they were marginally significant ($0.05 < P < 0.10$) (Table 6.6).

The distribution maps of band presences in relation to soil variables show that several bands (e.g. A183, B293, B320, B485 in relation to PbT,…) appear mainly in populations with low metal contents, and thus, those bands may not really be related to metals. However, two bands (B401 and B485) are present mainly in populations with higher soil contents of manganese. The map of B401 presences is presented in Fig. 6.5, whereas the maps for the other loci are in Supplementary Material S 6.4.

6.4 Discussion

6.4.1 Comparison of AFLP and cpSSR inferences of genetic diversity and differentiation

In this work we present a species-wide analysis of *C. ladanifer* using a genome scan of AFLP markers.

We have obtained an estimation of genetic differentiation ($\Phi_{ST} = 0.35$) which is much higher than data for other species (above the 3rd quartile in data from 77 species, Petit *et al.* 2005) and is congruent with the trends inferred by Nybom (2004) from species with similar life history traits (perennial, outcrosser, barochorous and from early-mid successional status).

Moreover, we estimated a quite low pollen/seed migration ratio, which indicates that the dispersal of seeds have accounted for a large component of the historical gene flow between populations (above the 45%). In fact, Metcalfe and

Table 6.6: Summary of results of the Mann-Whitney test on potentially environmental related loci detected by GEE. Only those loci with significant (or near significant) results are presented. ***U***: Mann-Whitney statistic. m_a and m_p refer to the median value of the related variable for the group of plants where the AFLP marker was absent or present, respectively. n_a and n_p refer to the number of plants where each AFLP marker was absent or present, respectively. ***P***: probability of obtaining a more extreme *U* value than that presented by chance alone.

Locus	*Related Variable*	*U*	m_a	n_a	m_p	n_p	*P* (2-tailed)
A183	SbT	5302.0	0.014	189	0.012	81	< 0.001
A189	pH	1101.5	6.30	250	6.99	50	< 0.001
A339	NiT	2626.5	45.99	240	27.86	30	0.016
A492	NiT	1417.5	45.99	255	18.69	15	0.092
B293	MnT	1273.0	532.7	252	241.9	16	0.013
B320	MnT	2677.0	532.7	238	424.8	30	0.026
B346	SbT	3688.0	0.012	229	0.014	39	0.082
B391	MnT	742.5	478.4	258	2805.0	10	0.001
B401	MnT	2614.5	454.9	233	1577.5	35	0.001
B485	MnT	1065.0	478.4	252	2191.3	16	0.002
B485	PbT	997.0	17.75	252	11.59	16	0.001

Kunin (2006) reported that pollination success in isolated *C. ladanifer* plants dropped to 0 when the distance to the nearest neighbour was around 3.5 m.

This near equal contribution of seed and pollen flow may explain the fact that, in spite of the different properties (such as mutation rate, ploidy level, homoplasy, inheritance) of AFLPs and cpSSRs, the matrices of inter-population genetic distances based on each type of markers were significantly correlated.

As a consequence of matrix correlations, similar NJ and Bayesian clusterings were obtained with both markers. The only discrepancy between markers was observed in the population EAL, located in a contact zone between different glacial lineages (Quintela-Sabarís *et al.* 2010).

We may consider this case as an example of the aforementioned properties of maternally-inherited cpDNA (e.g. Comes and Kadereit 1998), which conserve the history of colonisation, whereas in the case of nuclear markers this history is blurred by recombination or by pollen flow from nearby populations.

Similarities in population clustering also implies that AFLPs tell the same story as cpSSRs about the origin of the metallicolous (M) populations of *C. ladanifer*: they are the result of multiple and independent colonisation events that, in addition, did not imply any genetic differentiation related to soil type, as indicated by the AMOVA results within each BAPS-

defined cluster. Instead, and given the significance of the Mantel test on geographic and genetic distances, the genetic differentiation may be explained better as a result of historical processes (i.e. location and isolation of glacial refugia) together with isolation-by-distance. In this case, the significant difference between M and NM populations revealed by species-wide AMOVA can be interpreted as a spurious result produced by a "covariation" of phylogeography and soil type: each type of population has a different relative weight within each cluster inferred by BAPS. Hence, and following Staton *et al.* (2001), we have to underline the need to understand the phylogeography of a species as a prerequisite to correctly interpreting the effect of stress factors (heavy metals, in our case) on its genetic structure.

Similarly, studies which applied AFLPs to other pseudometallophytes also revealed the lack of an effect of soil type on genetic differentiation (e.g. *Cerastium velutinum*- Gustafson *et al.* 2003; *Onosma echiodes*- Mengoni *et al.* 2006; *Armeria maritima*- Baumbach and Hellwig 2007).

From a taxonomic point of view, and like previous cpSSR results (Quintela-Sabarís *et al.* 2010), the populations from S and SE Andalusia (Betic area) actually form a cluster separated from the other populations, nevertheless they are identified as belonging to subsp. *ladanifer* on the basis of leaf morphology. In contrast, the populations of the subsp. *sulcatus* (formerly *Cistus palhinhae*), despite showing several morphological differences, do not form a single cluster and are instead grouped together with populations of subsp. *ladanifer*.

Regarding subsp. *sulcatus*, our results are in contrast with those obtained by Carlier *et al.* (2008), who, analysing populations from the Algarve region (S of Portugal) with AFLPs and ISSRs, inferred a genetic differentiation between subsp. *ladanifer* and subsp. *sulcatus*. Our differences may be explained by the fact that Carlier *et al.* (2008) analysed DNA samples bulked for each population and also because the ISSR markers had an important effect, since they showed some alleles exclusive to one of the two subspecies considered. However, they obtained a low differentiation between subspecies (Dice index 0.98; Carlier *et al.* 2008).

Overall, these results point to the need for a taxonomic revision of the intraspecific taxa in *C. ladanifer*, especially in order to recognize the value and unique genetic features of Betic populations of this species.

As indicated by the AMOVA analyses, most of the genetic variation revealed by AFLPs occurs at the intrapopulation level, as would be expected for nuclear markers in an obligate-outcrossing species such as *C. ladanifer* (Loveless and Hamrick 1984, Nybom 2004). The assessment of genetic diversity within populations may suffer bias due to the dominant nature of AFLP markers. In order to overcome this possible bias, we have used SMC, the best metric for dominant markers in diploid species (Kosman and Leonard 2005), and we complemented SMC with two allele frequency-based diversity

estimators (PPL and Hj), computed using a robust Bayesian approach (Zhivotovsky 1999). Thus, we have obtained reliable estimators of genetic diversity, giving the similarities and significant correlations among the three indices.

As we previously inferred with cpSSRs, AFLPs detected no differences in genetic diversity related to soil type, a trend that seems to be common in the analyses of pseudometallophytes with nuclear-DNA markers (Mengoni *et al.* 2000, Mengoni *et al.* 2006, Baumbach and Hellwig 2007; but also some exceptions, e.g. Deng *et al.* 2007). This fact points to a lack of selective constraints on metallicolous populations of *C. ladanifer* and, thus, it may be an additional support on our consideration of tolerance to heavy metals as a constitutive trait in this species (Quintela-Sabarís *et al.* 2010).

However, we have to keep in mind that we are working with more than one hundred (putatively) independent and neutral markers, so possible selective effects in few markers may be diluted by the other neutral ones.

Ribeiro *et al.* (2002) found a correlation of genetic diversity estimations obtained with either AFLPs or cpSSRs in *Pinus pinaster*. These authors attributed this congruence to the fact that in this species gene flow through pollen (cpDNA is paternally-inherited in *P. pinaster*) is more important than marker-specific factors. In the case of *C. ladanifer*, the maternally-inherited cpDNA is not influenced by pollen flow, so the lack of correlation among the estimates of within-population genetic diversity obtained with AFLP markers and those obtained with cpSSRs, may be caused by the previously mentioned different properties of each kind of markers and the different effect of processes (such as genetic drift, recombination…) on them.

6.4.2 Detection of relevant loci using GEE

We have applied generalized estimating equations (GEE) to our data in order to reveal loci potentially related to soil variables (pH, Ca:Mg ratio, but especially heavy metal contents in soils). Contrary to other methods for detecting loci under selection, which usually rely on prior assumptions about population structure, migration and mutation rates, and also involve the estimation of F_{ST} from allele frequencies (see Bonin *et al.* 2007, and references therein), GEE directly correlate the allele distribution of each marker independently with environmental variation to produce 'allele distribution models'. Thus, these models are also individual-based and hence insensitive to biases caused by low sample size (Holderegger *et al.* 2008), an issue especially interesting for our study, with a medium number of plants per population. Although we are analysing the relationships between soil conditions and AFLP, other two main factors may include "noise" on our analyses: phylogeography and climatic variation. We have corrected the possible influence of phylogeography by introducing a correcting factor based on the previous information about chloroplast lineages (Quintela-Sabarís *et al.* 2010). Regarding the climate, PCA analyses on

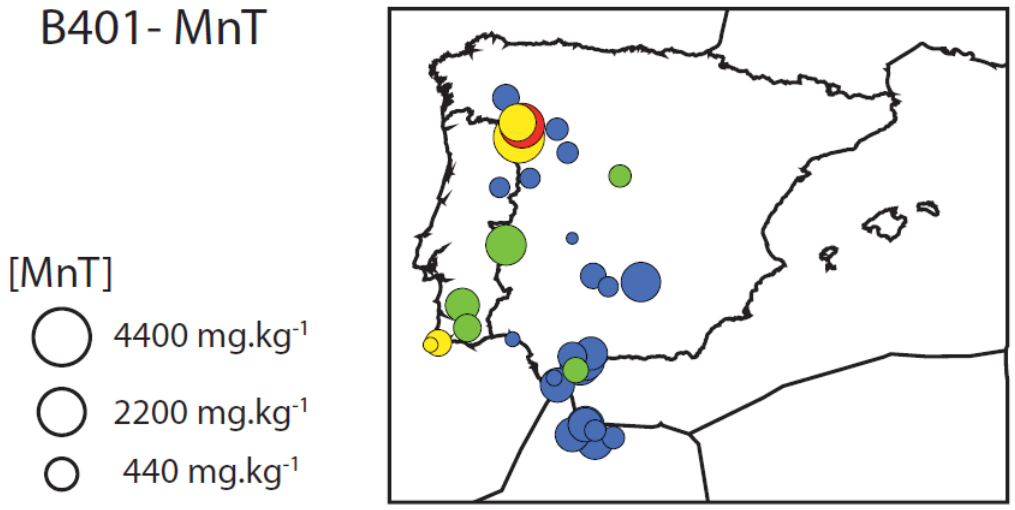


Figure 6.5: Geographic distribution of loci B401, potentially related to MnT. The geographic distribution of the band presence overlays the spatial variation of MnT. The diameter of each circle is proportional to the value of MnT in each population. The colour of the circle represent for each population the frequency (***f***) of plants in which the AFLP-fragment is present: blue (***f*** = 0); green (0 < ***f*** ≤ 0.50); yellow (0.50 < ***f*** ≤ 0.75) and red (0.75 < ***f***).

19 bioclimatic variables from each population revealed no clines or patterns on our populations (data not shown), so we may conclude that the allele distribution models mainly reflect the influence of soil variables.

Regarding soil variables, it is remarkable that we did not detected any loci related to Ca:Mg ratio, which points to a lack of selective effect of this soil variable on *Cistus ladanifer*. High levels of Mg in relation to Ca are considered one of the main stress factor to plants growing on serpentine soils (e.g. Brady *et al.* 2005). This unexpected result is congruent with previous reports signalling the low Ca requirements of *C. ladanifer* as a competitive trait allowing this plant growing in serpentine soils (Alados *et al.* 1999), and the high Mg requirements of *C. ladanifer* plants growing on serpentine soils from N of Morocco(Ater *et al.* 2000).

The opposite situation to Ca:Mg ratio is MnT, the total content of manganese in soils, which was shown to be the most influential of the soil variables. Its effect is even higher than other variables with a similar variation range (up to several thousand $mg \cdot kg^{-1}$ in soil; e.g. NiT and PbT), and may be related to the higher mobility in soils of Mn compared to Ni or Pb (Friedland 1990).

Manganese is a micronutrient essential to plant metabolism. It has functions in photosynthesis and also a protective role against oxidative stress (Epstein and Bloom 2005), but it may be toxic at high concentrations, provoking chlorosis

or necrosis. Thus, plants have to regulate the uptake of this element and its translocation in plant body in order to fulfil plant requirements but also in order to keep Mn levels below the threshold of safety for this metal (Wenzel *et al.* 2004).

Cistus ladanifer has been described in the literature as a Mn accumulator (Alvarenga *et al.* 2004, de la Fuente *et al.* 2010). Moreover, in a broadscale study, we have estimated that populations of *C. ladanifer* from the chloroplast lineage 'North' have higher levels of Mn in leaves and higher Mn leaf:soil ratios than populations from lineage 'South' (unpublished results), so this species must have developed mechanisms to regulate the uptake and translocation and thus, the tolerance to this metal, especially in some areas where the soil content of Mn is especially high (such as serpentine areas from NE Portugal or mine tailings from the C of Iberian Peninsula).

Among the loci whose distribution was significantly related to MnT, we have strong confidence in B401 being linked to genes involved in the tolerance to increasing levels of Mn. We support this assertion on the basis that i) the 'present' allele is more frequent in populations with high MnT values, and ii) these populations are distributed in a wide geographic area, thus avoiding local effects. Interestingly, most of the populations where B401 is present belong to cpLineage 'North', with higher Mn accumulation (see previous paragraph).

However, we are conscious of the fact that this does not prove of the adaptive relevance of marker B401, so, in accordance with Holderegger *et al.* (2008), subsequent analyses, which may include the isolation, sequentiation and identification of this band or the development of reciprocal transplant experiments, are needed in order to prove the adaptive or selective advantage of this band in relation to Mn.

In summary, we have obtained results that have allowed us to conclude that AFLPs provide similar inferences to those obtained by cpSSRs on the phylogeography and the colonisation of metalliferous areas by the pseudometallophyte *C. ladanifer* (multiple colonisation, independent origin, lack of genetic differentiation related to soil type), although there are some differences, related to the different properties of each kind of marker. In addition, we have proved the potentialities of GEE analysis, since it allowed us to estimate which soil variables explained more allele distributions in *C. ladanifer*, as well as to detect a band with a possible role in tolerance to high Mn concentrations in soils.

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Final Synthesis and Conclusions

Previous page: General view of a shrubland community growing on soils developed from metabasic rocks, near Vila Verde (Trás-Os-Montes, NE Portugal). This area was recently burned and *Cistus ladanifer* subsp. *ladanifer* has now become the dominant plant. (Photo: PS Kidd)

Final Synthesis and Conclusions

As was presented in previous chapters, we have conducted a series of studies on phylogeography, population genetics and ecophysiology of the Mediterranean pseudometallophytic species *Cistus ladanifer* (rockrose).

On the basis of a broad scale analysis of chloroplast microsatellites (cpSSRs), we have come to the conclusion that genetic diversity in this widespread species is geographically structured in two (or three, depending on the statistical methodology employed) main lineages or clusters.

These clusters are, in congruence with pollen data from the bibliography, the result of postglacial recolonisation of this species from isolated refugia located in N of Morocco, SW Iberian Peninsula and SE Spain.

Moreover, we have inferred that metallicolous populations of this species are the result of multiple and independent processes of colonisation. Interestingly, the colonisation of metalliferous areas has not left genetic imprints related to soil type (in the form of genetic bottlenecks or founder effects). This fact constitutes the first piece of evidence to support the theory that tolerance to heavy metals is a species-wide trait in rockrose.

There are a great number of local and regional studies on the accumulation of heavy metals by *C. ladanifer*. The application of phylogeographic knowledge allowed us to perform a study at a species-scale level, separating lineage and soil effects.

We have not observed different metal contents between metallicolous (M) and non-metallicolous (NM) populations of this species for a series of metals, except Ni. A plausible explanation may be the occurrence of mechanisms for restricted metal accumulation in M populations.

Notwithstanding the previous assertion, we have found different patterns of accumulation of heavy metals among M populations from different chloroplast lineages. This phenomenon, already observed in other pseudometallophytes, both reflects and supports the independent history of M populations, which have evolved in parallel within lineages that have been isolated since the Last Glacial Maximum.

However, and in spite the fact taht the species *C. ladanifer* clearly rejects the accumulation of the metals Co, Cr and Pb, we have noticed considerable differences among particular populations in response to other metals. This means that any phytostabilization procedure using this plantshould be preceded by a survey that allows the characterization of its local ecotypes in relation to heavy metals, in order

to prevent a rockrose-mediated transfer of metals into the ecosystems' food chain.

Our research carried out on tolerance to Co, Ni and Zn, in hydroponic cultures, revealed that each metal affected plants in a different way. Metal effects are congruent with the patterns of metals accumulation/exclusion we observed from field samples. Thus, for future analysis of metal tolerance in plants it would be useful to know the strategy of response to heavy metals in a given species in order to determine the best parameter to be measured.

Under the conditions of our hydroponics experiment we observed no differences in most response variables (growth, biomass, chlorophyll fluorescence) among M and NM populations. This fact may be interpreted as a second piece of evidence for the tolerance to metals as a constitutive (species-wide) trait in *C. ladanifer*.

Again, different chloroplast lineages also implied different patterns or mechanisms of response to metals. However, given the observed effects of metal treatments on response variables, we suggest that a possible preadaptation to nutrient shortage and water stress, instead of true metal tolerance, may have facilitated the colonisation of metalliferous soils by *C. ladanifer*.

Although the properties of AFLP markers differ with respect to cpSSRs (diploid, biparentally-inherited genome vs. haploid, maternally-inherited), they provided similar inferences on the phylogeography of the species. Moreover, AFLPs revealed no influence of soil type on the genetic diversity and differentiation of this species.

The GEE procedure was shown to be a useful statistical tool that allowed us to consider molecular data and soil information together. According to evidence provided by different authors on the nutrient requirements of *C. ladanifer*, we perceived no effect of Ca:Mg ratio (one of the most important factors in serpentine stress) on the distribution of AFLP markers. In contrast, we found that Mn content in soils has the strongest effect on allele distribution among the variables we analysed. In fact, we report the detection of a band with a possible role in tolerance to high Mn soil content, although future research in this area is needed.

Some final remarks to improve the present knowledge of *Cistus ladanifer*

Finally, we would like to suggest some future research lines in order to look for answers to questions that are still unresolved:

- ***Root symbioses and tolerance***: there is increasing evidence on the important role of soil microorganisms in the tolerance of plants to metals. In our case, this area deserves more attention, given the fact that *C. ladanifer* is a pseudometallophyte with several known ectomycorrhizal fungi and where some Plant Growth Promoting Rhizobacteria (PGPR) have been de-

scribed.

- ***Development of landscape genetic analysis using AFLP markers***: The Alentejo area (in fact, all the Iberian pyrite belt) provides an interesting scenario to study the evolution and adaptation to seriously stressing soil conditions at a landscape scale. As we have shown in this Ph.D. Thesis, allele distribution models on AFLP data provide potentially useful tools to advance in this area.

- ***Integrate phylogeography into chemoecology of the species***: leaf exudates provide beneficial effects to *C. ladanifer*. It has been proved that a seasonal and a regional variation in these exudates exists. It would be interesting to investigate whether isolation among chloroplast lineages also resulted in differences in exudates.

Complete Bibliography

This section comprises all the bibliographic references cited in the text (all chapters).

Previous page: *Cistus ladanifer* subsp. *ladanifer* var. *albiflorus* plants growing next to the Ni-hyperaccumulator *Alyssum serpyllifolium* (small-yellow flowers) on ultramafic outcrops in Samil (Trás-Os-Montes, NE Portugal). (Photo: PS Kidd)

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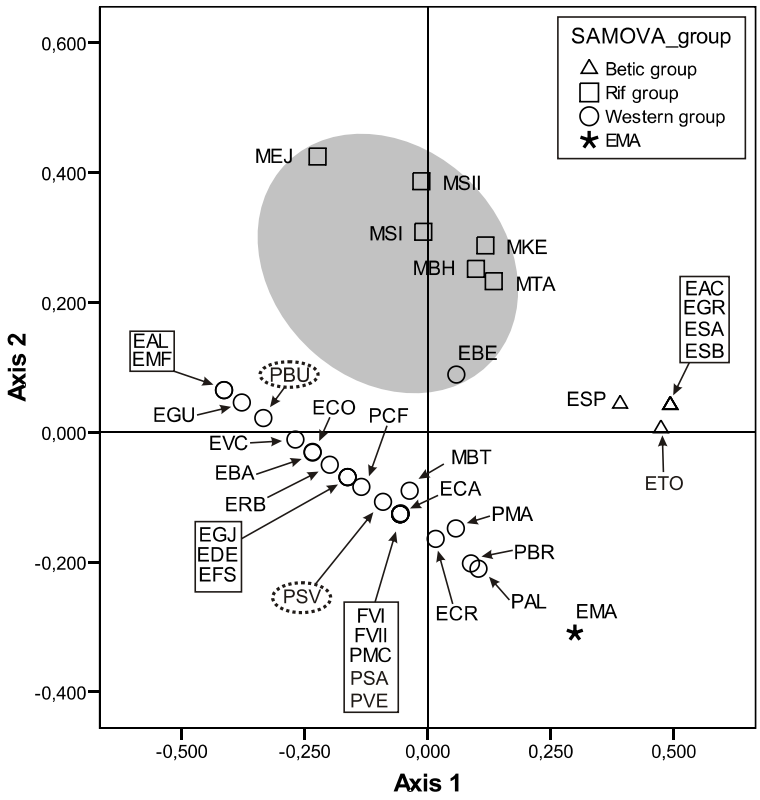
Supplementary material

This section includes tables and figures which complement the information presented in chapters 2-6.
Tables and figures are numbered using the format: **Supplementary material S *x.y***; where ***x*** is the chapter number and ***y*** is the order of the table/figure in the chapter ***x***.

Previous page: Drop of *labdanum* on a young stem of *Cistus ladanifer*, near Grazalema (Cádiz province, S of Spain). *Labdanum* provides protection against herbivores and also against excessive insolation. (Photo: C. Quintela-Sabarís)

Supplementary material S 2.1: Principal Coordinate Analysis (PCoA) results. Populations were plotted along the two first PCoA axes. For population codes, see Table 2.1. Different symbols are used for each SAMOVA-defined group. Grey shading indicates populations of *C. ladanifer* subsp. *africanus*. Population codes encircled by a dotted line indicate populations of *C. ladanifer* subsp. *sulcatus*. In some cases, population codes are connected to their plot by an arrow. Population codes inside squares indicate populations occupying exactly the same position.



Supplementary material S 2.2: Bibliographical sources of occurrences of *Cistus ladanifer* pollen. These sources were used to construct the map presented in Figure 2.4. First we present a table with a summary of each paper. Then, on next page, the complete bibliographical references are presented.

Reference	Site	Type of deposit	Long	Lat	Datation
Fletcher *et al.* (2007)	CM5 borehole (Guadiana Valley, Algarve, Portugal)	Sediment core	-7.45	37.27	First peak around 12,000 BP and another from 4,040 to 2,830 BP
Franco-Múgica *et al.* (2001)	Espinosa de Cerrato (Palencia, Spain)	River Franco marsh	-3.94	41.96	First peak around 8,000 BP and then new peaks from 2,700 BP to present times
Franco-Múgica *et al.* (2005)	El Carrizal lake (Segovia, Spain)	Sediment core from lake deposit	-4.15	41.32	Around 2,500 BP
López-Sáez *et al.* (2007)	Los Barruecos (Cáceres, Spain)	Archaeological site	-6.50	39.42	Around 6,000 BP
López-Sáez *et al.* (2009)	Portlligat Bay (Girona, Spain)	Sediment core from Posidonia oceanica living bed	3.29	42.29	Around 1,300 BP
Nocete *et al.* (2008)	Valencina de la Concepción (Guadalquivir Valley, Seville, Spain)	Archaelogical site, smelting quarter	-6.07	37.41	4,150 to 4,045 BP
Pons and Reille (1988)	Padul (Granada, Spain)	Radiocarbon dated peat core	-3.67	37.00	90,000 cal BP
Stevenson (2000)	Ojos del Tremedal (Montes Universales, Teruel, Spain)	Radiocarbon dated peat core	-2.05	40.54	1,830 to 440 cal BP
Van der Knaap and Van Leeuwen (1995)	Charco da Candieira (Serra da Estrela, Portugal)	Sediment core from lake deposit	-7.58	40.34	2,685 BP
Van der Knaap and Van Leeuwen (1997)	Lagoa Comprida 2 (Serra da Estrela, Portugal)	Sediment core from lake deposit	-7.64	41.32	Around 10,000 BP
Van der Schriek *et al.* (2007)	Muge River (Near Santarém, Portugal)	Sediment core in river bank	-8.66	39.1	From 5,800 to 5,200 cal BP

Longitude and *Latitude* are presented in decimal degrees. *BP*: years before present. *cal BP*: calibrated years before present

Supplementary material S 2.2: (continued)

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- López-Sáez JA, López-Merino L, Mateo MA, Serrano O, Pérez-Díaz S, Serrano L (2009) Palaeoecological potential of the marine organic deposits of *Posidonia oceanica*: A case study in the NE Iberian Peninsula. Palaeogeography, Palaeoclimatology, Palaeoecology 271:215-224
- Nocete F, Queipo G, Sáez R, Nieto JM, Inácio N, Bayona MR, Peramo A, Vargas JM, Cruz-Auñón R, Gil-Ibarguchi JI, Santos JF (2008) The smelting quarter of Valencina de la Concepción (Seville, Spain): the specialised copper industry in a political centre of the Guadalquivir Valley during the Third millennium BC (2750-2500 BC). Journal of Archaeological Science 35:717-732
- Pons A, Reille M (1988) The Holocene- and Upper Pleistocene pollen record from Padul (Granada, Spain): a new study. Palaeogeography, Palaeoclimatology, Palaeoecology 66:243-263
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- Van der Knaap WO, Van Leeuwen JFN (1995) Holocene vegetation succession and degradation as responses to climatic change and human activity in the Serra de Estrela, Portugal. Review of Palaeobotany and Palynology 89:153-211
- Van der Knaap WO, Van Leeuwen JFN (1997) Late Glacial and early Holocene vegetation succession, vegetation zonation, and climatic change in the Serra de Estrela, Portugal. Review of Palaeobotany and Palynology 97:239-285.
- Van der Schriek T, Passmore DG, Stevenson AC, Rolão J (2007) The palaeogeography of Mesolithic settlement-subsistence and shell midden formation in the Muge valley, Lower Tagus Basin, Portugal. The Holocene 17:369-385.

Supplementary material S 3.1: Total and extractable contents of metals in soils of the populations analysed in chapter 3. Population Codes and assigned Groups are the same as in table 3.1. Refer to Fig. 3.1 to see the ordination of the populations obtained by a Principal Component Analysis of metal contents. <*d.l.* indicates values under detection limit
4

Pop Code	Group	Total metal contents (µg.g^{-1})							Extractable metal contents (µg.g^{-1})						
		Co	Cr	Cu	Mn	Ni	Pb	Zn	Co	Cr	Cu	Mn	Ni	Pb	Zn
EAC	M(u)	89	2054	31	994	1893	33	62	68	1	7	670	183	12	<d.l.
EAL	M(h)	17	127	24	1119	33	13	70	14	0.55	11	930	11.5	9	9
EBE	NM	3	53	3	94	8	17	15	<d.l.	<d.l.	<d.l.	138	<d.l.	<d.l.	11
ECA	NM	6	48	4	216	58	33	26	<d.l.	<d.l.	<d.l.	152	<d.l.	<d.l.	5
ECO	M(h)	22	87	103	2127	57	43	85	12	<d.l.	68	2500	12	16	21
ECR	NM	3	33	5	210	0	28	33	<d.l.	<d.l.	<d.l.	430	<d.l.	15	14
EDE	M(h)	36	90	28	1824	37	174	123	28	<d.l.	9	2000	6	169	60
EFS	NM	5	48	3	242	14	18	20	<d.l.	<d.l.	<d.l.	161	<d.l.	<d.l.	<d.l.
EGJ	NM	24	120	29	455	46	49	123	<d.l.	<d.l.	<d.l.	264	<d.l.	<d.l.	<d.l.
EGR	NM	8	146	17	658	65	8	36	8	<d.l.	<d.l.	1030	10	<d.l.	5
EGU	NM	5	12	7	304	0	50	61	<d.l.	<d.l.	27	272	<d.l.	<d.l.	12
EMA	NM	3	79	32	80	19	14	15	<d.l.	<d.l.	18	24	<d.l.	6	5
EMF	NM	9	104	42	533	47	44	83	<d.l.	<d.l.	20	79	<d.l.	6	7,5
ERB	NM	7	70	7	273	3	65	28	8	<d.l.	<d.l.	400	<d.l.	91	17
ESB	M(u)	58	1518	23	525	715	17	81	12	0.6	<d.l.	133	67.5	10	8
ESP	M(u)	140	3204	24	1957	2914	27	84	62	0.75	<d.l.	740	250	5	9
ETO	M(u)	201	3803	21	1672	2963	32	58	181	0.95	<d.l.	1800	580	18	<d.l.
EVC	NM	3	34	11	34	3	16	16	<d.l.	<d.l.	<d.l.	61	<d.l.	<d.l.	<d.l.
MBH	NM	10	29	7	279	15	10	18	6	<d.l.	<d.l.	400	<d.l.	5	<d.l.
MBT	NM	21	178	27	1091	49	16	93	<d.l.	<d.l.	<d.l.	292	<d.l.	<d.l.	<d.l.
MEJ	NM	7	81	13	230	16	11	28	5	<d.l.	<d.l.	309	<d.l.	8	12
MKE	NM	22	122	40	1395	61	21	115	5	<d.l.	<d.l.	560	<d.l.	<d.l.	6
MSI	M(u)	88	2262	23	1201	1871	11	66	55	0.6	<d.l.	820	237	<d.l.	6
MSII	M(u)	123	2762	42	1598	2575	16	75	91	0.65	5	1070	350	<d.l.	<d.l.
MTA	NM	1	75	9	0	8	9	9	<d.l.	<d.l.	<d.l.	2	<d.l.	<d.l.	<d.l.
PAL	M(h)	17	75	269	1830	44	2168	892	40	<d.l.	75	2700	14	590	430
PBR	M(u)	69	944	56	1578	1151	4	76	5	<d.l.	<d.l.	140	8	<d.l.	<d.l.
PBU	NM	12	49	7	478	12	14	30	8	<d.l.	<d.l.	700	8	<d.l.	<d.l.
PCF	NM	21	79	24	602	51	16	55	5	<d.l.	<d.l.	650	5	<d.l.	<d.l.
PMC	M(u)	220	9248	47	4362	4573	18	170	89	<d.l.	5	1400	451	<d.l.	8
PSA	M(u)	150	6191	155	2805	1543	9	121	48	0.38	18	635	175.5	<d.l.	<d.l.
PSV	NM	2	30	5	69	11	5	8	<d.l.	<d.l.	<d.l.	75	<d.l.	<d.l.	5
PVE	NM	2	16	7	209	0	25	68	<d.l.	<d.l.	<d.l.	33	<d.l.	<d.l.	<d.l.

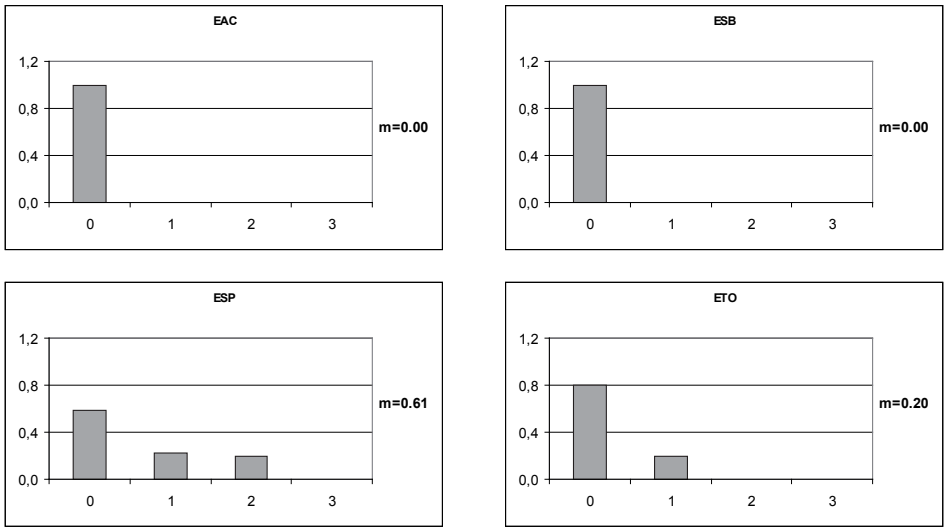
Supplementary material S 3.2: Definition of cpSSR haplotypes (chlorotypes) detected in *Cistus ladanifer*. Haplotype codes are the same as in Figure 3.2.

Allele Size (bp)		***Haplotype Code***	***N***
ccmp2	*ccmp3*		
136	113	**H1**	6
136	114	**H2**	20
136	115	**H3**	109
136	116	**H4**	72
136	117	**H5**	1
137	114	**H6**	46
137	115	**H7**	46
137	116	**H8**	21
138	115	**H9**	2
138	116	**H10**	1

N: number of plants displaying the haplotype

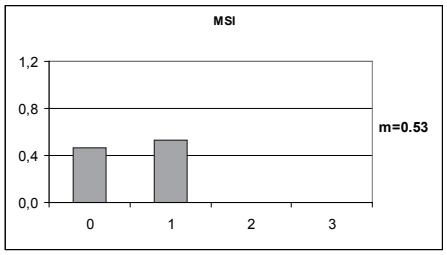
Supplementary material S 3.3: Distribution of pairwise chloroplast simple sequence repeats (cpSSR) length differences among plants. A graph is presented for each population (for population codes see table 3.1). Populations were arranged according to their chloroplast lineage ('North' or 'South') and their soil type (Metallicolous or Non-metallicolous). Ordinates represent the frequency; abscissa, the length difference. In addition, the mean difference (***m***) is indicated for each histogram.

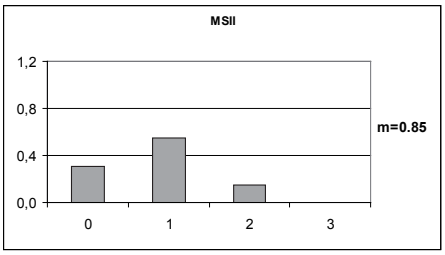
'South' Lineage, Metallicolous Populations



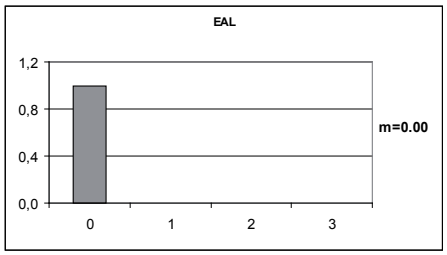
Supplementary material S 3.3: (continued)

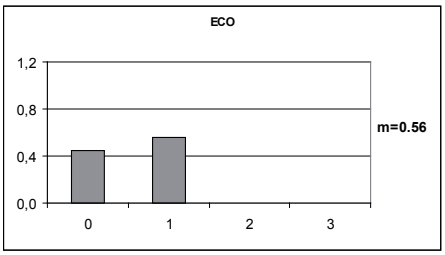
'South' Lineage, Metallicolous Populations

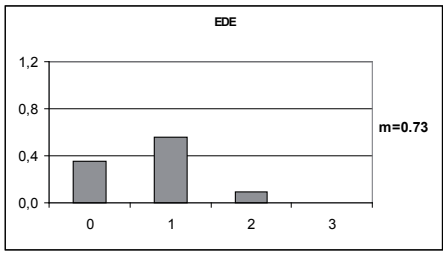


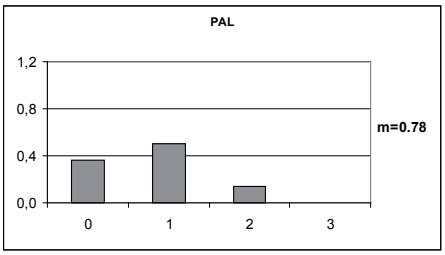


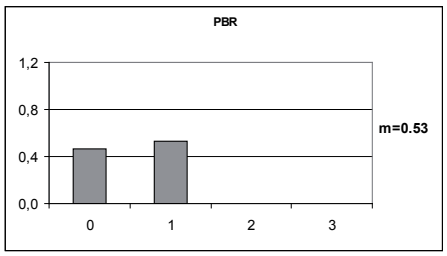
'North' Lineage, Metallicolous Populations

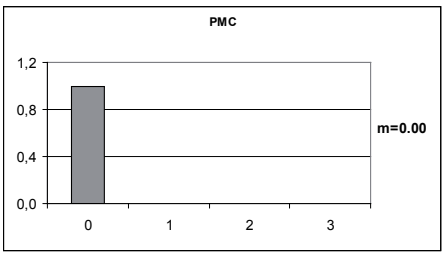


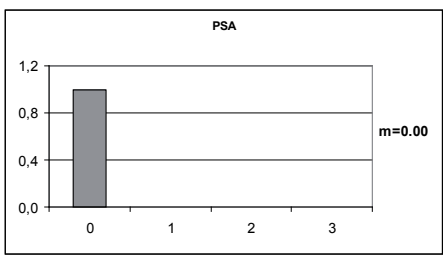




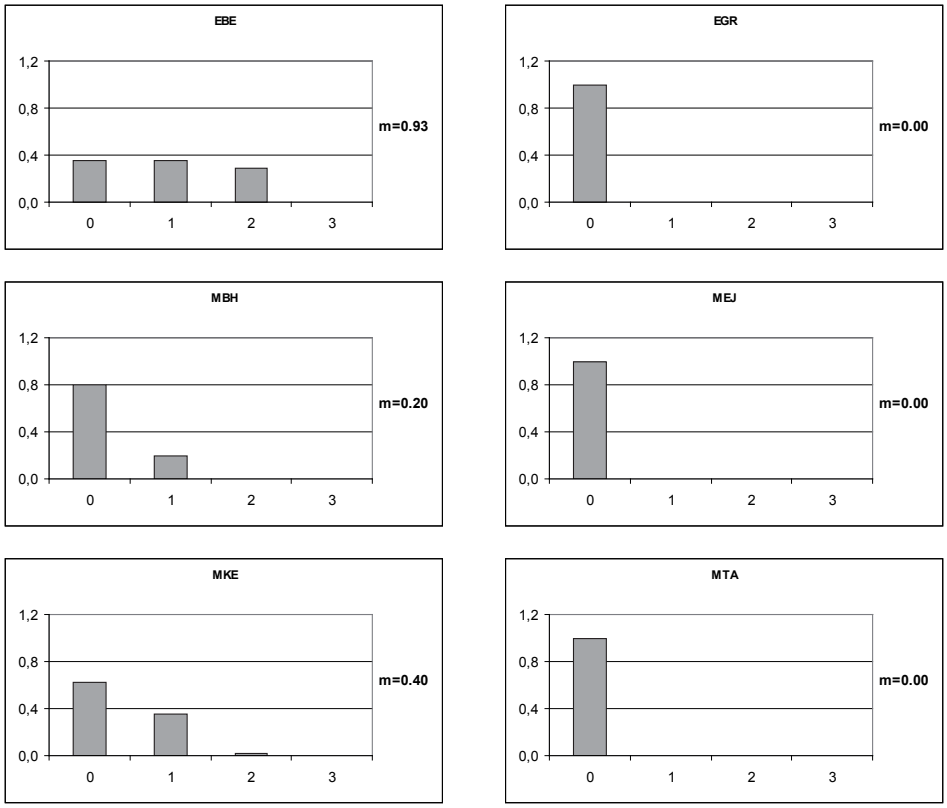




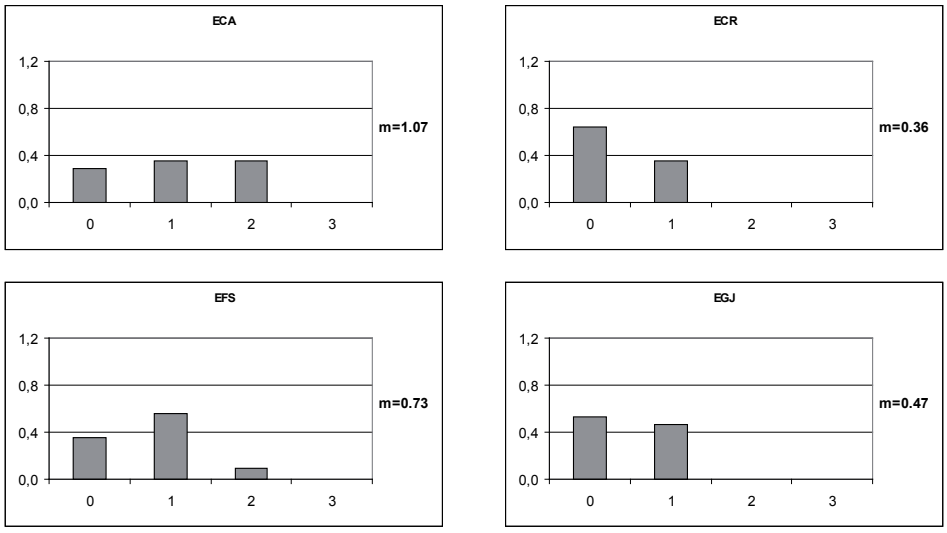




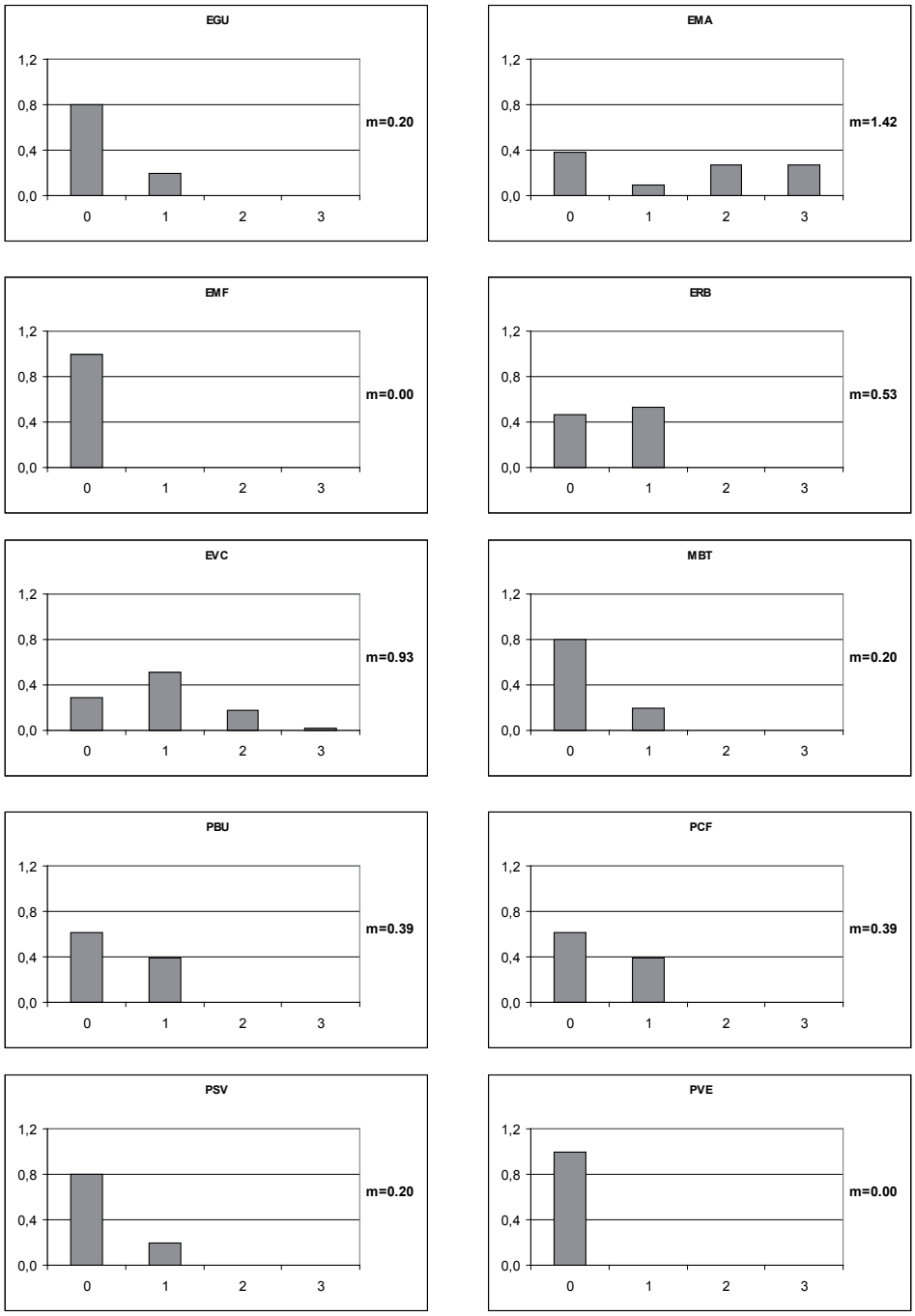
Supplementary material S 3.3: (continued)
'South' Lineage, Non-metallicolous populations



'North' Lineage, Non-metallicolous Populations



Supplementary material S 3.3: (continued)
'North' Lineage, Non-metallicolous Populations



Supplementary material S 5.1: Total and extractable contents of Co, Ni and Zn in soils of the populations included in the experiments of tolerance in hydroponic cultures. Pop Codes, and assigned Group and Lineages are the same as in table 5.1. Refer to chapter 3 in order to see the justification of Groups and Lineages.

Pop Code	Group	Lineage	Total metal contents (µg.g^{-1})			Extractable metal contents (µg.g^{-1})		
			Co	Ni	Zn	Co	Ni	Zn
EAC	M	South	89	1893	62	68	183	<d.l.
EBE	NM	South	3	8	15	<d.l.	<d.l.	11
ECA	NM	North	6	58	26	<d.l.	<d.l.	5
ECO	M	North	22	57	85	12	12	21
ECR	NM	North	3	0	33	<d.l.	<d.l.	14
EGJ	NM	North	24	46	123	<d.l.	<d.l.	<d.l.
EMA	NM	North	3	19	15	<d.l.	<d.l.	5
EMF	NM	North	9	47	83	<d.l.	<d.l.	7,5
ERB	NM	North	7	3	28	8	<d.l.	17
ESB	M	South	58	715	81	12	67,5	8
ESP	M	South	140	2914	84	62	250	9
ETO	M	South	201	2963	58	181	580	<d.l.
MBT	NM	North	21	49	93	<d.l.	<d.l.	<d.l.
MKE	NM	South	22	61	115	5	<d.l.	6
MSI	M	South	88	1871	66	55	237	6
MSII	M	South	123	2575	75	91	350	<d.l.
PAL	M	North	17	44	892	40	14	430
PBR	M	North	69	1151	76	5	8	<d.l.
PBU	NM	North	12	12	30	8	8	<d.l.
PCF	NM	North	21	51	55	5	5	<d.l.
PMA	NM	North	5	9	10	<d.l.	<d.l.	<d.l.
PMC	M	North	220	4573	170	89	451	8
PSA	M	North	150	1543	121	48	175,5	<d.l.
PSV	NM	North	2	11	8	<d.l.	<d.l.	5
PVE	NM	North	2	0	68	<d.l.	<d.l.	<d.l.

Extractable contents refer to Ammonium Acetate/EDTA/Acetic Acid, pH 4.65 ***<d.l.*** indicate values under detection limit

Supplementary material S 6.1: Summary of soil variables used for Principal Component Analysis. Data presented are pH, total and extractable Ca:Mg ratios, and total contents of metals As and Sb The total and extractable contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn are presented in Supplementary material S 3.1.

Pop Code	pH	Ca:Mg ratio		Metal contents (μg·g^{-1})	
		Total	Extractable	As	Sb
EAC	6.77	0.05	0.43	4.81	0.02
EAL	7.93	4.34	39.3	5.24	0.01
EBE	4.85	2.29	5.51	3.57	0.01
ECA	5.82	0.7	8.23	9.57	0.01
ECO	7.26	1.92	19.53	54.27	4.51
ECR	6.62	1.29	5.5	8.43	0.03
EDE	6.5	2.24	10.78	27.05	0.03
EFS	6.53	1.18	3.75	7.83	0.02
EGJ	5.8	0.09	2.82	26.31	0.01
EGR	6.92	2.3	20.61	9.68	0.01
EGU	6.31	1.67	6.85	1.51	0.01
EMA	6.01	5.91	11	11.31	0.11
EMF	5.41	2.23	9.59	30.71	0.06
ERB	5.41	2.25	12.57	6.78	0.03
ESB	5.98	0.19	0.89	7.87	0.01
ESP	5.06	0.04	1.86	1.21	0.01
ETO	6.99	0.02	0.79	3.93	0.01
EVC	4.96	1.48	6.55	9.55	0.07
MBH	6.58	0.98	12.22	3.51	0.01
MBT	5.18	0.17	1.86	4.4	0.01
MEJ	6.05	2.54	8.18	2.56	0.01
MKE	6.3	0.82	10.04	6.98	0.01
MSI	7.1	0.1	1.37	3.82	0.01
MSII	7.17	0.12	0.71	4.59	0.01
MTA	5.16	0.48	1.01	9.78	0.01
PAL	4.22	1.23	8.74	752.45	1.10
PBR	6.02	0.23	1.91	3.36	0.01
PBU	6.51	0.93	9.14	14.49	0.01
PCF	5.13	0.1	4.23	11.25	0.01
PMC	6.6	0.19	0.45	5.52	0.02
PSA	6.47	0.82	0.73	1.62	0.01
PSV	7.31	12.63	45.18	5.11	0.04
PVE	5.11	0.84	4.96	6.54	0.03

Supplementary material S 6.2: Matrix of population pairwise genetic distances. Below diagonal: Nei's D (alter Lynch and Milligan 1994) computed on AFLP data. Above diagonal: Cavalli-Sforza and Edwards (1967) distance computed on cpSSR data. cpSSR data were taken from chapter 3.

	EAC	EAL	EBE	ECA	ECO	ECR	EDE	EFS	EGJ	EGR	EGU	EMA	EMF	ERB	ESB	ESP
EAC		0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0	0.9	0.9	0.9	0.9	0	0.309
EAL	0.021		0.744	0.546	0.487	0.9	0.546	0.546	0.605	0.9	0.204	0.669	0	0.546	0.9	0.9
EBE	0.017	0.008		0.569	0.611	0.61	0.558	0.558	0.606	0.9	0.673	0.731	0.744	0.606	0.9	0.693
ECA	0.047	0.042	0.033		0.438	0.507	0.261	0.261	0.476	0.9	0.458	0.647	0.546	0.453	0.9	0.831
ECO	0.053	0.044	0.037	0.000		0.546	0.207	0.207	0.131	0.9	0.293	0.546	0.487	0.064	0.9	0.787
ECR	0.053	0.045	0.037	0.000	0.002		0.428	0.428	0.452	0.9	0.762	0.697	0.9	0.499	0.9	0.754
EDE	0.044	0.036	0.030	0.000	0.000	0.001		0	0.224	0.9	0.378	0.57	0.546	0.206	0.9	0.787
EFS	0.059	0.052	0.043	0.013	0.010	0.011	0.008		0.224	0.9	0.378	0.57	0.546	0.206	0.9	0.787
EGJ	0.040	0.035	0.023	0.003	0.007	0.006	0.003	0.008		0.9	0.418	0.556	0.605	0.067	0.9	0.765
EGR	0.013	0.010	0.004	0.033	0.043	0.036	0.035	0.046	0.022		0.9	0.9	0.9	0.9	0	0.309
EGU	0.060	0.054	0.041	0.012	0.015	0.014	0.012	0.019	0.001	0.040		0.593	0.204	0.355	0.9	0.852
EMA	0.059	0.054	0.041	0.003	0.006	0.002	0.005	0.013	0.002	0.039	0.002		0.669	0.548	0.9	0.831
EMF	0.052	0.047	0.035	0.006	0.010	0.006	0.010	0.017	0.000	0.031	0.006	0.001		0.546	0.9	0.9
ERB	0.057	0.051	0.039	0.000	0.002	0.000	0.003	0.010	0.001	0.038	0.003	0.000	0.000		0.9	0.775
ESB	0.019	0.018	0.008	0.034	0.038	0.040	0.032	0.044	0.024	0.003	0.044	0.043	0.034	0.040		0.309
ESP	0.011	0.018	0.006	0.029	0.038	0.032	0.031	0.040	0.016	0.000	0.033	0.032	0.025	0.031	0.005	
ETO	0.002	0.016	0.009	0.030	0.038	0.034	0.030	0.042	0.021	0.001	0.039	0.037	0.029	0.035	0.006	0.000
EVC	0.053	0.044	0.033	0.010	0.009	0.009	0.010	0.015	0.001	0.033	0.004	0.007	0.000	0.002	0.033	0.027
MBH	0.045	0.041	0.024	0.049	0.054	0.053	0.045	0.061	0.032	0.033	0.051	0.056	0.049	0.053	0.031	0.026
MBT	0.045	0.044	0.030	0.002	0.006	0.007	0.002	0.017	0.004	0.033	0.009	0.004	0.011	0.002	0.034	0.027
MEJ	0.047	0.052	0.030	0.053	0.062	0.057	0.051	0.066	0.038	0.036	0.059	0.058	0.053	0.058	0.037	0.028
MKE	0.040	0.044	0.020	0.044	0.051	0.048	0.044	0.054	0.028	0.024	0.047	0.049	0.041	0.047	0.024	0.018
MSI	0.052	0.059	0.036	0.059	0.069	0.063	0.059	0.071	0.043	0.040	0.061	0.064	0.057	0.063	0.044	0.035
MSII	0.045	0.049	0.029	0.053	0.063	0.053	0.052	0.066	0.037	0.032	0.057	0.057	0.050	0.056	0.037	0.029
MTA	0.059	0.065	0.044	0.063	0.070	0.068	0.058	0.078	0.049	0.047	0.069	0.068	0.061	0.067	0.042	0.043
PAL	0.034	0.040	0.022	0.024	0.028	0.028	0.025	0.035	0.008	0.019	0.018	0.024	0.017	0.022	0.017	0.010
PBR	0.045	0.049	0.031	0.029	0.033	0.036	0.028	0.040	0.014	0.031	0.028	0.033	0.023	0.028	0.026	0.023
PBU	0.049	0.052	0.034	0.039	0.041	0.046	0.038	0.050	0.021	0.036	0.035	0.044	0.033	0.038	0.026	0.028
PCF	0.045	0.048	0.028	0.031	0.031	0.039	0.031	0.042	0.017	0.031	0.032	0.039	0.028	0.033	0.022	0.023
PMC	0.055	0.057	0.040	0.026	0.032	0.031	0.031	0.035	0.014	0.036	0.018	0.023	0.014	0.019	0.039	0.031
PSA	0.054	0.057	0.038	0.031	0.036	0.034	0.033	0.039	0.017	0.037	0.022	0.030	0.020	0.024	0.035	0.029
PSV	0.043	0.045	0.031	0.020	0.020	0.027	0.018	0.029	0.013	0.032	0.026	0.027	0.018	0.023	0.023	0.024
PVE	0.064	0.059	0.046	0.017	0.017	0.016	0.019	0.016	0.015	0.047	0.028	0.020	0.015	0.013	0.044	0.039

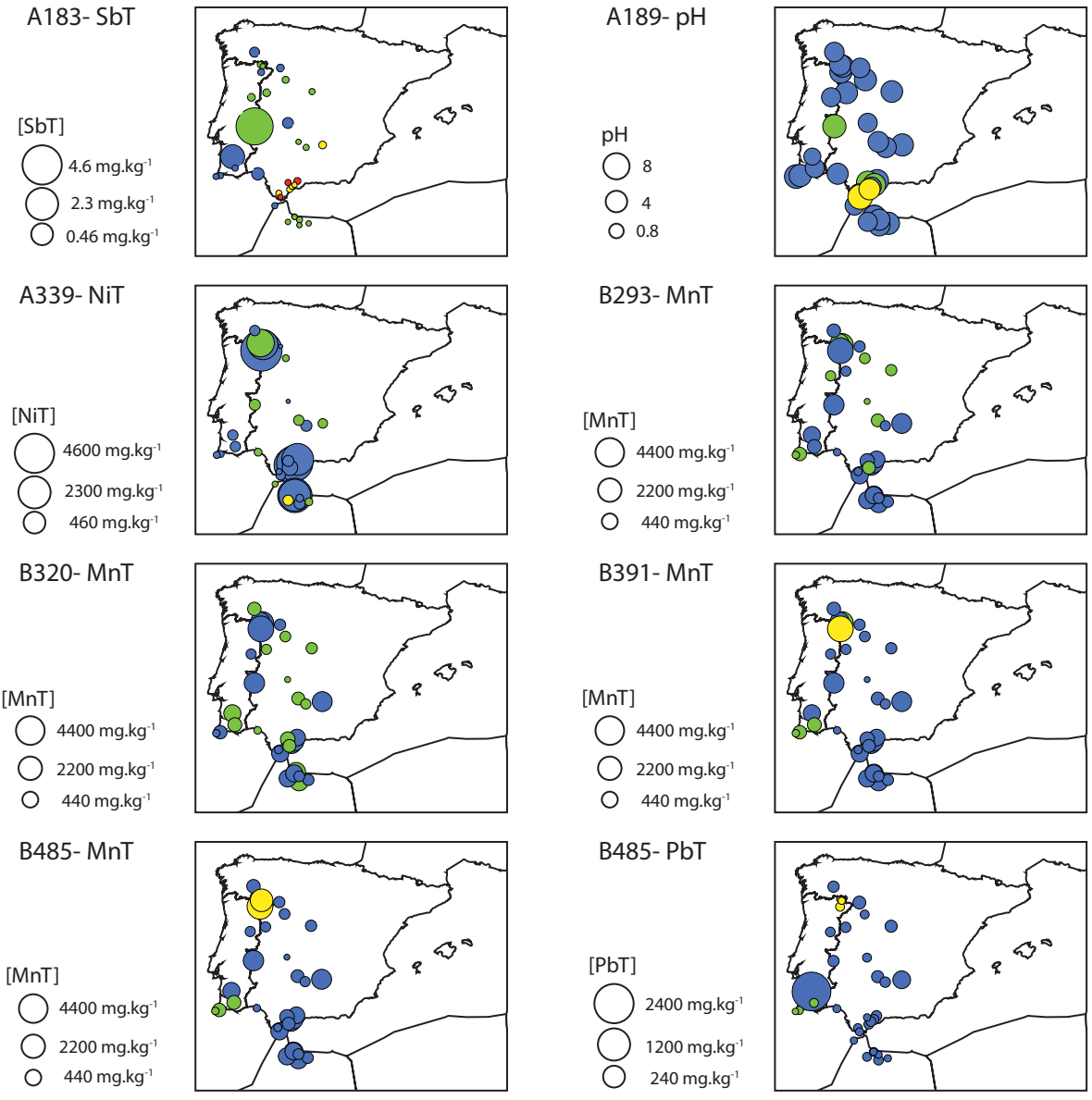
Supplementary material S 6.2: (continued)

	ETO	EVC	MBH	MBT	MEJ	MKE	MSI	MSII	MTA	PAL	PBR	PBU	PCF	PMC	PSA	PSV	PVE
EAC	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
EAL	0.9	0.487	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.735	0.9	0.309	0.655	0.9	0.9	0.744	0.9
EBE	0.854	0.591	0.464	0.518	0.9	0.499	0.569	0.637	0.427	0.57	0.606	0.643	0.608	0.669	0.669	0.621	0.669
ECA	0.805	0.296	0.9	0.683	0.9	0.9	0.9	0.9	0.9	0.22	0.453	0.433	0.499	0.669	0.669	0.552	0.669
ECO	0.9	0.302	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.537	0.605	0.187	0.187	0.487	0.487	0.293	0.487
ECR	0.834	0.547	0.9	0.35	0.9	0.9	0.9	0.9	0.9	0.349	0.14	0.685	0.414	0.293	0.293	0.35	0.293
EDE	0.854	0.23	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.343	0.452	0.297	0.252	0.487	0.487	0.324	0.487
EFS	0.854	0.23	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.343	0.452	0.297	0.252	0.487	0.487	0.324	0.487
EGJ	0.9	0.354	0.9	0.409	0.9	0.9	0.9	0.9	0.9	0.521	0.534	0.315	0.056	0.364	0.364	0.164	0.364
EGR	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
EGU	0.9	0.356	0.9	0.753	0.9	0.9	0.9	0.9	0.9	0.637	0.782	0.108	0.471	0.744	0.744	0.569	0.744
EMA	0.9	0.596	0.9	0.683	0.9	0.9	0.9	0.9	0.9	0.693	0.728	0.566	0.566	0.669	0.669	0.593	0.669
EMF	0.9	0.487	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.735	0.9	0.309	0.655	0.9	0.9	0.744	0.9
ERB	0.9	0.323	0.9	0.464	0.9	0.9	0.9	0.9	0.9	0.526	0.569	0.25	0.123	0.427	0.427	0.23	0.427
ESB	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
ESP	0.364	0.814	0.744	0.685	0.9	0.754	0.775	0.798	0.735	0.809	0.775	0.827	0.756	0.735	0.735	0.744	0.735
ETO		0.854	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.787	0.805	0.9	0.9	0.9	0.9	0.9	0.9
EVC	0.032		0.9	0.624	0.9	0.9	0.9	0.9	0.9	0.415	0.552	0.31	0.386	0.605	0.605	0.456	0.605
MBH	0.030	0.043		0.753	0.744	0.204	0.23	0.386	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MBT	0.027	0.016	0.042		0.9	0.762	0.782	0.803	0.744	0.605	0.464	0.669	0.364	0.204	0.204	0.285	0.204
MEJ	0.031	0.053	0.008	0.042		0.744	0.546	0.536	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MKE	0.023	0.038	0.003	0.037	0.006		0.295	0.321	0.293	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MSI	0.037	0.055	0.013	0.050	0.008	0.005		0.273	0.427	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MSII	0.031	0.049	0.006	0.048	0.005	0.003	0.001		0.536	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MTA	0.045	0.063	0.028	0.059	0.027	0.031	0.036	0.029		0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
PAL	0.016	0.015	0.024	0.021	0.030	0.020	0.037	0.033	0.042		0.257	0.593	0.52	0.585	0.585	0.53	0.585
PBR	0.026	0.021	0.029	0.026	0.038	0.026	0.044	0.041	0.042	0.001		0.717	0.507	0.427	0.427	0.464	0.427
PBU	0.032	0.027	0.031	0.037	0.042	0.031	0.048	0.045	0.043	0.004	0.000		0.37	0.655	0.655	0.471	0.655
PCF	0.028	0.021	0.026	0.031	0.041	0.026	0.045	0.043	0.043	0.001	0.000	0.000		0.309	0.309	0.108	0.309
PMC	0.034	0.012	0.048	0.028	0.054	0.042	0.057	0.052	0.063	0.008	0.007	0.013	0.013		0	0.204	0
PSA	0.034	0.015	0.039	0.031	0.049	0.035	0.054	0.049	0.056	0.002	0.000	0.005	0.004	0.000		0.204	0
PSV	0.026	0.017	0.034	0.022	0.041	0.031	0.047	0.046	0.046	0.006	0.000	0.004	0.000	0.008	0.004		0.204
PVE	0.044	0.019	0.056	0.022	0.062	0.053	0.070	0.063	0.066	0.031	0.032	0.043	0.033	0.035	0.036	0.021	

Supplementary material S 6.3: Results of GEE analyses in *Cistus ladanifer*. All loci which correlated at least with one soil variable in the sampling area are given. QIC is the quasi-likelihood under the independence model criterion for GEE. The best models were of the kind of $\beta_0 + \beta_1 \cdot Variable_1 + \beta_2 \cdot Variable_2$.

Locus	QIC	β_0	$Variable_1$	β_1	$P\text{-value}_1$	$Variable_2$	β_2	$P\text{-value}_2$
A153	78.1	$3.95 \cdot 10^{15}$	**PbT**	$-8.71 \cdot 10^{11}$	0			
A183	319.6	-2.4	**SbT**	-0.23	$4.20 \cdot 10^{-7}$			
A189	112.8	-12.61	**pH**	1.52	$8.66 \cdot 10^{-7}$	**ZnT**	0.03	$1.29 \cdot 10^{-5}$
A245	286.1	-1.78	**SbT**	0.23	$4.03 \cdot 10^{-12}$			
A339	198.5	1.6	**NiT**	-0.002	$7.09 \cdot 10^{-6}$			
A492	123.3	-2.66	**NiT**	-0.002	$1.31 \cdot 10^{-6}$			
B152	121.5	4.6	**NiT**	-0.002	0.003			
B169	202.2	-1.0	**MnT**	0.002	$6.25 \cdot 10^{-8}$	**ZnT**	-0.04	$8.03 \cdot 10^{-5}$
B217	66.2	3.60	**SbT**	-0.60	$3.74 \cdot 10^{-11}$			
B293	122.9	-2.90	**MnT**	-0.002	0.001			
B320	187.1	-2.07	**MnT**	-0.002	0.001	**ZnT**	0.02	$5.69 \cdot 10^{-4}$
B346	238.4	-1.44	**SbT**	0.30	$3.24 \cdot 10^{-9}$			
B391	43.0	-3.1	**MnT**	0.001	0.001	**SbT**	-47.13	$8.95 \cdot 10^{-9}$
B401	178.2	-2.28	**MnT**	0.001	$4.43 \cdot 10^{-7}$			
B450	76.0	3.7	**SbT**	-63.64	$2.86 \cdot 10^{-5}$			
B485	70.0	-1.7	**MnT**	0.001	0.002	**PbT**	-0.09	$1.74 \cdot 10^{-4}$

Supplementary material S 6.4: Geographic distribution of loci potentially related with soil variables. For each locus we present a map where the geographic distribution of the band presence overlays the spatial variation of the soil variable with which it is correlated. The diameter of each circle is proportional to the value of the concerned soil variable in each population. The colour of the circle represents for each population the frequency (***f***) of plants in which the AFLP-fragment is present: blue (***f*** = 0); green (0 < ***f*** ≤ 0.50); yellow (0.50 < ***f*** ≤ 0.75) and red (0.75 < ***f***). Only bands which resulted in a Mann-Whitney test with a ***P*** lower than 0.05 are shown in the maps.



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Durante parte do desenvolvimento desta tese fum beneficiario dumha Bolsa de Pré-Doutoramento da Xunta de Galiza.

Departamento de Botánica
Facultade de Bioloxía
Universidade de Santiago de Compostela

Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L.

Celestino Quintela Sabarís
Tese de Doutoramento, Marzo 2011

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE BOTÁNICA
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Dra. **M. ISABEL FRAGA VILA**, PROFESORA TITULAR DE UNIVERSIDADE DO DEPARTAMENTO DE BOTÁNICA DA FACULTADE DE BIOLOXÍA DA UNIVERSIDADE DE SANTIAGO DE COMPOSTELA,

INFORMA:

Que a presente memoria titulada "**Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L."** presentada por **D. Celestino Quintela Sabarís** para optar ó Grao de Doutor en Bioloxía, foi realizada baixo a miña dirección no Departamento de Botánica da Universidade de Santiago de Compostela.

E considerando que representa traballo de Tese de Doutoramento, autorizo a súa presentación ante o Tribunal correspondente.

E para que así conste, asino a presente en Santiago de Compostela a 22 de Marzo de 2011.

Vº e Prace da Directora,
Asdo.: Dra. M. Isabel Fraga Vila

O Doutorando,
Asdo.: D. Celestino Quintela Sabarís

Aos meus pais e avós, responsáveis polo autor
A Berta e Martinho, que me lembram cada dia o que é importante na vida
A Bibi.

Contents

RESUMO EN GALEGO DA TESE DE DOUTORAMENTO
Orixe evolutiva e ecofisioloxía das poboacións metalícolas de *Cistus ladanifer* L.

Introdución e antecedentes:

Os solos metalíferos (aqueles que conteñen cantidades elevadas de metais) son tóxicos para a maioría das plantas e outros organismos vivos. Dentro dos solos metalíferos, aqueles resultado da polución por actividades humanas, especialmente por actividades relacionadas coa produción de metais (entulleiras de minas, áreas de fundición...) constitúen unha ameaza para o medio ambiente e a saúde pública: normalmente teñen unha escasa (ou mesmo ausente) cobertura vexetal e, polo tanto, os metais poden sofrer unha filtración cara as augas subterráneas, ou o propio solo poluído pode dispersarse polo vento e afectar terras agrícolas produtivas ou áreas protexidas (Ruttens *et al.* 2006, Tordoff *et al.* 2000).

Un resultado desta preocupación pública foi a promulgación pola Comisión Europea da Estratexia Europea Temática para a protección dos Solos (COM (2006) 231) que inclúe unha proposta de directiva marco sobre os solos. Un dos principais obxectivos desta estratexia é a recuperación de solos degradados, sendo a contaminación por metais pesados unha das causas principais da degradación do solo. Estímase que no seo da UE, existen preto de 0,5 millóns de lugares contaminados que precisan ser recuperados (SEC (2006) 1165).

As tecnoloxías clásicas para a recuperación de solos adoitan implicar procesos que son caros e moitas veces destrutivos (solidificación e estabilización, lavado do solo, electrocinética, redución/oxidación química, incineración, vitrificación, escavación e eliminación en vertedoiro, etc) (Wenzel *et al.* 2004).

Porén, o uso de plantas para a recuperación de lugares contaminados con metais (Fitorremediación) está gañando unha importancia considerábel, xa que a fitorremediación é considerada "menos invasiva e máis eficaz na restauración da estrutura do solo e das funcións en comparación cos métodos de enxeñería civil" (Kidd *et al.* 2009).

Centrándonos no caso dos metais pesados, a base de todas estas fitotecnoloxías é a existencia, identificación e descrición de especies vexetais (ou poboacións dentro de determinadas especies), que sexan tolerantes a metais pesados. É dicir, plantas que teñan a capacidade de colonizar, sobrevivir e reproducirse nun solo con elevadas concentracións de metais pesados, tóxico para a maioría das outras plantas (Antonovics *et al.* 1971, Macnair 1993).

Baker (1981) estableceu que, de acordo co seu patrón de resposta aos

metais, as especies tolerantes pódense clasificar en tres grupos principais: acumuladoras, indicadoras e exclusoras.

Así, unha exclusora presenta baixos niveis de metais na súa parte aérea para ampla gama de concentracións externas de metais, até un valor crítico no solo a partir do cal o mecanismo de exclusión quebra, o que resulta nunha falta de restricción do transporte do metal cara a parte aérea. En contraste, as especies acumuladoras concentran os metais na parte aérea, haxa unha concentración alta ou baixa de metal no solo, mentres que as plantas indicadoras mostran unha relación proporcional entre os niveis de metais no solo e a súa acumulación na parte aérea. Unha categoría adicional comprende as especies hiperacumuladoras: plantas que se comportan de forma semellante ás acumuladoras, mais cun factor de acumulación moito máis elevado.

Desde o punto de vista da súa distribución respecto ás áreas con metais pesados, podemos clasificar as especies vexetais en tres tipos (Pollard *et al.* 2002): non-metalófitos estrictos (especies restrinxidas a solos con baixo contido de metais, que é o caso da gran maioría das plantas), metalófitos estrictos (plantas que medran exclusivamente en solos metalíferos, como entulleiras de minas ou áreas serpentínicas) e metalófitos facultativos, tamén chamados pseudometalófitos (especies con poboacións que medran en solos metalíferos e non metalíferos).

Dentro dos pseudometalófitos podemos distinguir dous subtipos: por unha banda, as especies con tolerancia constitutiva (todos os xenotipos son tolerantes a metais, mesmo naquelas poboacións que medran en solos con baixo contido de metais) e do outro lado, as especies con variedades ou ecotipos tolerantes aos metais que evolucionaron durante a colonización de áreas metalíferas a partir de poboacións onde os xenotipos tolerantes estaban presentes en baixa frecuencia (Pollard *et al.* 2002).

Para completar este esquema, relativamente simple, tamén se ten citado o caso de especies vexetais que, con independencia da súa tolerancia a metais, grazas a posuír preadaptacións a algunha das condicións edáficas estrictas dos solos metalíferos (falta de nutrientes ou desequilibrio nos mesmos, estrés hídrico, alta insolación, etc) poden colonizar estas áreas máis facilmente (Brady *et al.* 2005; e referencias citadas) de xeito que, tal como Wu (1990) describiu, é posíbel a colonización de solos metalíferos sen a evolución da tolerancia.

De cara á posta en práctica de procesos de fitorremediación, os pseudometalófitos posúen un elevado interese, xa que alén da súa tolerancia aos metais, posúen outras características importantes, como son a súa alta capacidade de adaptación a unha ampla gama de condicións adversas do solo, xunto coa súa elevada produción de biomasa e a súa competitividade en solos con moderada toxicidade por metais (Poschenrieder *et al.* 2001).

Alén disto, e dadas as diferenzas en tolerancia que podemos topar entre poboacións dentro dunha especie de pseudometalófito, estes organismos permíten-

nos investigar a xenética e fisioloxía das diferenzas entre as plantas tolerantes e non tolerantes dunha forma que xeralmente non é posíbel cos edafo-endemismos (Macnair 1993). Ademais, as poboacións metalícolas (M) e non-metalícolas (NM) veciñas dun determinado pseudometalófito son modelos relevantes para o estudo da adaptación local en plantas (Linhart e Grant 1996).

A esteva ("gum-rockrose" en inglés ou"jara pringosa"en castellano-español) (*Cistus ladanifer* L., Fam Cistaceae) é un arbusto leñoso presente na Rexión Mediterránea Occidental (desde o sur de Francia até o Norte de Marrocos e Alxeria) (Demoly e Montserrat 1993).

Un exemplar adulto de *C. ladanifer* pode acadar os 2 m de altura, con sistemas radicais e aéreos densos (Martín Bolaños e Guinea López 1949). Posúe follas lanceoladas verdes cunha cara glabra e o envés cuberto cun tomento branco. As follas presentan unha disposición decusada e están soldadas entre sí pola base. A morfoloxía foliar emprégase como base para a identificación das súas tres subespecies: subspp. *africanus*, *ladanifer* e *sulcatus* (Demoly e Montserrat 1993). A subsp. *africanus* é a única con follas pecioladas, namentres nas subspp. *ladanifer* e *sulcatus* son sésiles. Ademais, na subsp. *sulcatus* (orixinalmente descrita como *Cistus palhinhae*) a superficie da folla está dividida por nervios ben marcados.

A subsp. *ladanifer* distribúese principalmente na Península Ibérica, norte de África e Francia (onde é considerada unha especie introducida), subsp. *sulcatus* é endémica para o sudoeste de Portugal (rexión do Algarve) e subsp. *africanus* está presente no sur de España (Cádiz, Málaga), pero é máis común no norte de África (Demoly e Montserrat 1993). Guzmán e Vargas (2009) dataron a orixe de *C. ladanifer* e mais a diverxencia das súas diferentes subespecies durante o Pleistoceno Superior.

As súas flores son grandes (55-70 mm de diámetro) e solitarias, con 3 sépalos e 5 pétalos brancos (var. *albiflorus*) que tamén pode ter unha mancha de cor vermella a acastañada na base (var. *maculatus*). Cada flor ten un estigma sésil e un gran número de estames que poden producir máis de 700.000 grans de pole por flor (Talavera *et al.* 1993).

A polinización é entomófila, e posúe un mecanismo gametofítico de autoincompatibilidade, polo que a esteva é unha especie alógama obrigada (Talavera *et al.* 1993). Este feito, xunto cunhas distancias de dispersión de pole curtas (poucos metros) resulta nunha redución da eficiencia reprodutiva en exemplares isolados (Metcalfe e Kunin 2006). O froito é unha cápsula loculicida, con 5-7 a 12 lóculos. Unha única cápsula pode conter até 1.000 sementes, polo que se estima que un adulto de *C. ladanifer* pode producir máis de 158.000 sementes, que se liberan durante un período de 8 a 10 meses desde mediados do verán (Bastida e Talavera 2002). A dispersión das sementes é principalmente barócora, e máis do 80% da caída de sementes ocorre baixo a copa das plantas nai (Bastida e Talavera 2002), aínda que se ten estimado que diferentes

especies de formigas granívoras (xénero *Goniomma*) e o cervo (*Cervus elaphus*), que se alimentan de sementes e froitos respectivamente, poden desempeñar un papel importante na dispersión de sementes a longa distancia (Bastida e Talavera 2002, Malo e Suárez 1998).

As poboacións de *C. ladanifer* constitúen as etapas iniciais da sucesión ecolóxica nos ecosistemas mediterráneos. Estas poboacións están adaptadas ás alteracións destes ecosistemas, especialmente ao lume: as súas sementes manteñen unha elevada viabilidade durante varios anos (sementes de 3 anos de idade amosaron unha taxa de xerminación por riba do 80%, observación persoal, C. Quintela-Sabarís) e posúen un mecanismo de dormición física que pode ser interrompida polo lume, as altas temperaturas (Pérez-García 1997), o fume ou as sales nitroxenadas (Pérez Fernández e Rodríguez Echeverría 2003).

Durante o primeiro ano post-incendio, ocorre unha xermolación masiva de plántulas (Ferrandis *et al.* 1999), que permite a rápida rexeneración das poboacións orixinais (dous anos despois dunha queima experimental, *C. ladanifer* cobre o 40% da área orixinal, Calvo *et al.* 2005).

Ademais, esta especie está ben adaptada ao estrés hídrico e ao exceso de insolación. Posúe un sistema radical denso e superficial que favorece a captación de auga nas limitadas épocas de chuvia (Martín Bolaños e Guinea López 1949). Alén disto, é semi-decídua, mantendo durante o período de seca estival follas de pequeno tamaño potencialmente activas (Núñez- Olivera *et al.* 1996). As follas tamén contan cunha fotoprotección grazas á exudación dunha resina perfumada e pegañenta (o ládano), que aumenta durante o verán (Chaves *et al.* 1993).

Dado o seu contido en flavonoides e outros compostos fenólicos do metabolismo secundario, a exudación de ládano ten efectos positivos adicionais na esteva: o ládano é un composto con propiedades alelopáticas que inhibe a xermolación doutras plantas (Herranz *et al.* 2006) e proporciona unha defensa contra a inxestión por parte de herbívoros ao inhibir neles a relaxación dos músculos esqueléticos da boca (Sosa *et al.* 2004).

En relación aos solos, *C. ladanifer* é o principal compoñente de matogueiras desenvolvidas en solos ácidos oligotróficos da metade occidental da Península Ibérica (Rivas-Martínez 1979), se ben modelos de distribución apoian a súa tolerancia a solos calcáreos (Gastón *et al.* 2009) e a subsp. *sulcatus* está restrinxida a solos derivados de caliza no litoral sudoeste de Portugal.

A súa capacidade de medrar en áreas pobres en nutrientes pode ser favorecida polo establecemento de relacións simbióticas con microorganismos nas súas raíces. Varias cepas de bacterias relacionadas coa solubilización de fosfato ou a produción de sideróforos (un deles identificado como rizobacteria promotora de crecemento vexetal- PGPR), foron illadas a partir de raíces de *C. ladanifer* (Ramos Solano *et al.* 2006). Ademais, na literatura científica existe referencia da identificación de máis de 30 especies de fungos que forman

relacións simbióticas (ectomicorrizas) con *C. ladanifer* (Comandini *et al.* 2006).

Pero o máis importante entre todas estas características interesantes é o feito de que ***C. ladanifer* é un pseudometalófito**. As subespecies *ladanifer* e *africanus* teñen establecido con éxito poboacións en áreas serpentínicas ou en entulleiras de mina onde, nalgúns casos, son a especie dominante. A presenza de *C. ladanifer* sobre solos metalíferos, e a acumulación de metais en follas desta planta está reflectida nunha ampla produción científica. Nalgúns destes traballos (Alvarenga *et al.* 2004, Pratas *et al.* 2005, Murciego Murciego *et al.* 2006), a esteva foi descrita como especie indicadora ou mesmo acumuladora de As, Sb e Zn, e Mn, Sb e W, respectivamente.

Estas referencias proporcionan información útil sobre a esteva e os metais, aínda que, ao ser estudos a nivel local ou rexional, mesmo empregando diferentes metodoloxías de mostraxe e cuantificación de metais, é difícil facer comparacións entre eles.

No relativo á tolerancia de *C. ladanifer* aos metais, só temos constancia de tres traballos científicos previos, cada un deles usando diferentes enfoques.

• Alados *et al.* (1999), usando unha análise de estabilidade no desenvolvemento, demostraron a adaptación de *C. ladanifer* aos solos serpentínicos de Málaga (S de España). Estes autores presentan a hipótese de que o baixo requerimento de Ca^{2+} por parte desta especie pode ser unha vantaxe na colonización de áreas serpentínicas. Nesta mesma liña, Ater *et al.* (2000) cuantificaron unha ratio Mg/Ca elevada en follas de *C. ladanifer* crecendo en zonas serpetínicas do norte de Marrocos.

• Kidd *et al.* (2004) someteron a plántulas de 5 de poboacións de solos metalíferos e non metalíferos do NE de Portugal a experimentos de tolerancia ao Cd, Co, Cr, Mn, Cu, Ni, Pb e Zn en cultivo hidropónico. Observaron patróns de tolerancia e acumulación de metais específicos para cada poboación, e estimaron que mesmo poboacións non-metalícolas posuían unha certa tolerancia aos metais pesados.

• Santos *et al.* (2009) procuraron actividade diferencial de enzimas antioxidantes en plantas de *C. ladanifer* dunha zona de minas abandonadas en SE Portugal, pero non atoparon ningunha variación relacionada especificamente cos metais.

Nunha Tese de Doutoramento de elevado interese, Díez-Lázaro (2008) tratou a optimización do uso de *Cistus ladanifer* para procesos de fitorremediación. Encontrou que a adición de fertilizantes e a acidificación do solo melloraron o crecemento e a extracción de Mn e Zn por plantas desta especie procedentes do Nordeste de Portugal. Ademais, estableceu que a esteva podería ser utilizado para a fitoextracción de Zn en solos con contido baixo-medio deste metal.

Finalmente, o efecto beneficioso desta planta é subliñado por Simões *et al.* (2009), quen estimaron que pode producir máis de 4.600 kg de follada·ha^{-1}·ano^{-1}, o que mellora a calidade do solo e pode promover a rexeneración da vexetación ao facilitar a colonización dunha área por es-

pecies con requerimentos máis esixentes.

En resumo, *C. ladanifer* posúe unha serie de características (adaptación á seca, baixo requerimento de nutrintes, tolerancia a metais en certas poboacións) que a fan especialmente útil para a recuperación de áreas degradadas na rexión mediterránea. Ademais, é unha especie nativa desta rexión rica en biodiversidade e, polo tanto, o seu uso non implica efectos prexudiciais sobre os ecosistemas circundantes producido polo emprego de especies exóticas e invasoras (Méndez e Maier 2008, e referencias citadas nese traballo). Alén disto, *C. ladanifer* constitúe unha especie modelo interesante para o estudo do proceso de colonización de áreas metálicos por plantas.

OBXECTIVOS E TAREFAS DESENVOLVIDAS:

Dentro deste marco, e co obxectivo de mellorar o coñecemento sobre *Cistus ladanifer* e as súas relacións con metais, desenvolveuse unha campaña de recollida de mostras de planta (follas, sementes) e de mostras de solo en diferentes localidades do rango desta especie (Rexión Mediterránea Occidental, principalmente Península Ibérica e Norte de Marrocos). Buscouse cubrir diferentes tipos de material xeolóxico, dando especial atención ás áreas metalíferas (solos serpentínicos de Trás-Os-Montes, Málaga e Rif; áreas mineiras do SW da Península Ibérica), resultando nun total de 33 poboacións. O material recolectado empregouse nunha serie de investigacións, desenvolvidas a fin de tratar os seguintes temas:

• Un primeiro paso para inferir os efectos dos metais sobre a xenética da especie é entender a interacción de procesos que determinan a súa filoxeografía ou "paisaxe xenética". Dentro dos diferentes tipos de marcadores moleculares, aqueles baseados no ADN do cloroplasto (cpADN, ben sexan microsatélites do cloroplasto-cpSSRs ou PCR-RFLPs) teñen sido amplamente empregados en estudos de filoxeografía de plantas (Petit *et al.* 2003, Magri *et al.* 2007) ao posuír unha serie de características útiles:

- Nas anxiospermas, o cpADN polo xeral hérdase por vía materna, polo que a súa dispersión realízase soamente através das sementes, resultando nunha mellor estruturación xeográfica das súas variedades.

- Ao herdarse por vía materna, os patróns xeográficos do cpADN non se ven influídos polo fluxo de pole entre poboacións.

- Ademais, o cpADN é haploide, polo que o seu tamaño efectivo de poboación é menor que o dos xenes nucleares (diploides). Destes xeito, a diferenciación por deriva xenética pode ser máis forte (Comes e Kadereit 1998) e fenómenos como colos de botella xenéticos poden ser detectados de xeito máis doado (Echt *et al.* 1998) co cpADN que cos xenes nucleares. Por exemplo, os marcadores cpSSR detectaron que as poboacións de *Silene paradoxa* medrando en entulleiras de minas de cobre sufriran unha redución na súa diversidade xenética (Mengoni *et al.* 2001), en canto un estudo

previo con RAPD sobre esas mesmas poboacións fallara na detección desa baixa diversidade (Mengoni *et al.* 2000).

- Finalmente, os microsatélites do cloroplasto son considerados marcadores neutros, é dicir, todos os seus alelos (variantes) teñen efectos iguais sobre o individuo que os transporta. Así, os cpSSRs proporcionar unha información independente da selección que nos permite separar os efectos da filoxeografía dos efectos da contaminación por metais.

É por isto que desenvolvemos un estudo a grande escala da filoxeografía de *C. ladanifer* empregando cpSSRs (capítulo 2).

• Posteriormente cuantificamos, empregando fluorescencia de raios X (XRF), ICP-masas e espectrometría de absorción atómica (AAS), os contidos totais e extraíbeis de metais nos solos de 33 poboacións procedentes de case todo o rango de *C. ladanifer*. En base a esta información, clasificamos esas poboacións como metalícolas (que medran en solos con contidos elevados de metais, abreviado M) e non-metalícolas (que medran en solos "normais", abreviado NM). O tipo de poboación integrouse coa información filoxeográfica dos cpSSRs (ver capítulo 3) para respostar ás seguintes preguntas: As poboacións M teñen unha orixe mono ou polifilética? A colonización de áreas metalíferas provoca perda de diversidade xenética?

• Un seguinte paso foi cuantificar a acumulación de metais pesados en follas de esteva recollidas no campo e procedentes das 33 poboacións consideradas no capítulo anterior; para o que empregamos XRF, ICP-OES e ICP-masas. Dado que a nivel xenético unha especie non é unha mestura homoxénea de alelos, senón que adoita estar dividida en subgrupos, no caso de que as poboacións M teñan unha orixe evolutiva múltiple, é posíbel que existan diferencias nas estratexias de resposta aos metais (Gonnelli *et al.* 2001, Nyberg Berglund *et al.* 2003). Consideramos este aspecto ao computar índices de bioacumulación de metais en cada poboación e avaliar o efecto do tipo de solo (metalífero ou non metalífero) e a información filoxeográfica sobre os patróns de acumulación (contidos totais e relativos de metais nas follas) (ver capítulo 4).

• Alén da resposta en acumulación en campo, desenvolvemos experimentos de invernadoiro en condicións de cultivo hidropónico para avaliar a tolerancia de *C. ladanifer* aos metais Co, Ni e Zn (ver capítulo 5). Estimamos a tolerancia en base a medidas de crecemento (lonxitude da raíz mais longa, lonxitude do caule e incremento de número de follas), biomasa (peso seco) e eficiencia fotosintética ("yield"). Transformamos estas variábeis en medidas relativas computadas como unha porcentaxe dos valores obtidos para as plantas control, segundo a proposta orixinal de Wilkings (1978) para o crecemento da raíz. Así, eliminamos a maior parte da variación nas respostas non relacionadas cos tratamentos con metais.

• Un último paso, interesante para o desen-

volvemento de futuras investigacións, é a identificación de marcadores potencialmente ligados á tolerancia a solos metalíferos. Isto é especialmente necesario en plantas non-modelo, como *C. ladanifer*. Abordamos este tema aproveitando as posibilidades de desenvolvemento de escáneres do xenoma ("genome scans") dos AFLP (siglas do inglés: Amplified Fragment Length Polymorphism) (Vos *et al.* 1995). Estes marcardores moleculares fornecen información do xenoma nuclear e permiten obter centos de marcadores potencialmente non ligados dunha especie determinada sen ter un coñecemento previo das secuencias do seu ADN. Os AFLPs teñen sido empregados en organismos non-modelo (Bonin *et al.* 2007, e referencias dese traballo). En concreto, utilizáronse para detectar loci potencialmente implicados na adaptación a diversas condicións ambientais en plantas (Namroud *et al.* 2008, Parisod e Christin 2008, Poncet *et al.* 2010), e mesmo adaptación a metais pesados en poboacións do pseudometalófito *Arabidopsis halleri* (Meyer *et al.* 2009). Nesta tese de doutoramento, optimizamos un protocolo de análise de *C. ladanifer* con AFLP, obtendo un elevado número de marcadores. Posteriormente, comparamos estes marcadores coa información dispoñíbel sobre os solos (pH, ratios Ca:Mg e contidos de metais pesados), mediante a aplicación de Ecuacións de Estimación Xeralizadas (GEE, en inglés) (ver capítulo 6). As GEE, que son unha extensión dos modelos lineares xeralizados (Carl e Kuhn 2007), permiten analizar os modelos de distribución de alelos e estimar as variábeis ambientais que teñen unha maior influencia sobre eses modelos de distribución. Ademais, as GEE permiten introducir e corrixir a posíbel autocorrelación entre as mostras procedentes dunha mesma poboación ou dunha mesma liñaxe cloroplastidial. Finalmente, realizamos unha comparación das inferencias da estrutura xenética das poboacións de *Cistus ladanifer* obtidas con AFLPs (marcadores nucleares, diploides, de herdanza biparental e que poden sufrir recombinación) e cpSSRs (marcadores cloroplastidiais, haploides, de herdanza materna, que non sofren recombinación e entón hérdanse como bloques-haplotipos).

SÍNTESE E CONCLUSIÓNS:

A seguir presentamos unha síntese final e conclusións elaboradas en base aos resultados obtidos a partir dos experimentos expostos nos parágrafos anteriores:

Grazas á análise a grande escala de *Cistus ladanifer* empregando microsatélites de cloroplasto (cpSSRs), inferimos que a diversidade xenética desta especie con grande rango de distribución está na realidade estructurada en dúas (ou tres, dependendo da metodoloxía estatística empregada) liñaxes ou clusters principais.

Atendendo aos datos de pole presentes na bibliografía, estes conxuntos son o resultado da recolonización posglacial da Rexión Mediterránea Occidental a partir de refuxios illados e situados no norte de Marrocos e no sudoeste e sueste da Península Ibérica. Así mesmo, temos que

concluír que as poboacións metalícolas desta especie son o resultado de múltiples e independentes procesos de colonización. Curiosamente, a colonización de áreas metalíferas non deixou pegadas xenéticas (na forma de colos de botella xenéticos ou efecto fundador) relacionadas co tipo de solo. Este feito constitúe un primeiro apoio á tolerancia a metais pesados como unha característica 'constitutiva' (presente en toda a especie) da esteva.

Hai un gran número de estudos locais e rexionais sobre a acumulación de metais pesados por *C. ladanifer*. A aplicación dos coñecementos filoxeográficos permitiunos separar, nun estudo a nivel de toda a especie, os efectos da liñaxe cloroplastidial e do solo. Ao compararmos as poboacións metalícolas (M) e non-metalícolas (NM), non observamos diferenzas entre elas nos contidos foliares de diferentes metais, agás Ni. Unha explicación plausible é a aparición, nas poboacións M, de mecanismos de restricción da acumulación de metais. A pesar da afirmación anterior, atopamos diferentes patróns de acumulación de metais pesados entre as poboacións M de liñaxes cloroplastidiais diferentes. Este fenómeno, xa observado noutras especies de pseudometalófitos, reflicte e serve de apoio a unha historia de evolución independente das poboacións M, que evolucionaron en paralelo dentro de liñaxes que se mantiveron illadas desde o Último Máximo Glacial (que rematou aprox. 20.000 anos antes do presente).

Con todo, e a pesar da esteva rexeitar claramente a acumulación dos metais Co, Cr e Pb, temos notado diferenzas significativas na resposta a outros metais entre as poboacións a nivel individual. Disto podemos derivar que calquera procedemento de fitoestabilización que implique o uso desta especie debe ser precedido por unha investigación que permita a caracterización dos seus ecotipos locais respecto dos metais pesados, a fin de evitar unha transferencia de metais (mediada pola esteva) do solo cara a rede trófica do ecosistema.

Os experimentos de tolerancia a Co, Ni e Zn, en condicións de cultivo hidropónico, revelaron que cada metal afecta de xeito diferente á esteva. Ademais, os efectos de cada metal son congruentes cos patróns de acumulación/exclusión que observamos para cada metal a partir de mostras tomadas no campo. Así, en futuras análises de tolerancia a metais en plantas sería útil coñecer a estratexia de resposta aos metais pesados dunha determinada especie, a fin de determinar o mellor parámetro (estimador de tolerancia) a ser medido.

Nas condicións do noso experimento non foron observadas diferenzas entre as poboacións M e NM para a meirande parte das variábeis resposta (crecemento, biomasa, fluorescencia clorofílica). Este feito pode ser interpretado como un segundo apoio para a tolerancia a metais como un carácter constitutivo de *C. ladanifer*. Unha outra vez, as diferentes liñaxes cloroplastidiais implican diferentes patróns ou mecanismos de resposta aos metais. Con todo, e dado o efecto dos tratamentos con metais sobre as variábeis resposta, suxerimos que pre-adaptacións á

escaseza de nutrientes ou ao estrés hídrico, en lugar dunha verdadeira tolerancia aos metais, poden ter facilitado a colonización de solos metalíferos por *C. ladanifer*.

Os marcadores AFLP, aínda posuíndo diferentes propiedades que os cpSSRs (xenoma diploide de herdanza biparental fronte a xenoma haploide de herdanza materna) forneceron as mesmas inferencias sobre a filoxeografia da especie. Así mesmo, os AFLPs non demostraron unha influencia do tipo de solo sobre a diversidade xenética e a diferenciación entre poboacións desta especie.

O procedemento de GEE resultou ser unha ferramenta estatística útil que nos permitiu analizar en conxunto os datos moleculares e a información sobre o solo. De acordo coas evidencias proporcionadas por diferentes autores sobre as esixencias nutricionais de *C. ladanifer*, a relación Ca: Mg (un dos factores de estrés máis importantes nas áreas serpentínicas, Brady *et al.* 2005) non tivo ningún efecto sobre a distribución de marcadores AFLP. Porén, verificamos que, entre todas as variábeis consideradas, o contido de Mn en solos ten o efecto máis forte na distribución de alelos. De feito, temos detectado unha banda cun posíbel papel na tolerancia ao alto contido de Mn no solo, aínda que precisamos futuras investigacións que permitan estimar o valor adaptativo da mesma.

Como apartado adicional, suxírense algunhas posíbeis liñas de futuras investigacións, que se verían favorecidas polos coñecementos derivados desta tese de doutoramento: i) estudo da implicación de simbioses planta-microorganimos (fungos ectomicorrízicos, rizobacterias) na tolerancia; ii) estudos sobre a colonización de áreas metalíferas a nivel da xenética da paisaxe e iii) integración dos datos filoxeográficos en estudos de quimioecoloxía (variación nos exudados foliares da esteva).

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Chapter 1

Evolutionary origin and ecophysiology of metallicolous populations of *Cistus ladanifer* L.: Introduction and objectives.

Previous page: Close-up of a flower of *Cistus ladanifer* subsp. *ladanifer* var. *maculatus* (with a spot on each petal), growing on ultramafic soils in Samil (Trás-Os-Montes, NE Portugal). Flowers of *C. ladanifer* produce high amounts of pollen and nectar that attract a diverse array of insects including beetles, flies and bees. (Photo: PS Kidd)

CHAPTER 1

Introduction and Objectives

Soils with high concentrations of metals (metalliferous soils) are toxic to most plants and other living organisms (Boxes 1.1 and 1.2). Moreover, soils polluted by human activities, particularly those activities related to the production of metals (mine tailings, smelting areas ...) constitute a threat to the environment and public health: they usually have sparse (or even absent) plant cover and thus metals can filter into groundwater or the polluted soil can be dispersed by wind and then affect productive agricultural land or natural reserves (Ruttens *et al.* 2006, Tordoff *et al.* 2000).

A result of this public concern is the promulgation of the European Thematic Strategy for Soil Protection (COM (2006) 231) by the European Commission, which includes a proposal for a Framework Directive on Soils. One of the main objectives of this Strategy is the restoration of degraded soils, with pollution due to heavy metals being one of the causes of soil degradation. It is estimated that within the EU there are around 0.5 million contaminated sites which need to be remediated (SEC (2006) 1165). Classic technologies for the remediation of soils involve processes that are expensive and often destructive (solidification and stabilization, soil flushing, electrokinetics, chemical reduction/oxidation, soil washing, incineration, vitrification, excavation/retrieval, landfill and disposal, etc) (Wenzel *et al.* 2004).

In contrast, the use of plants for the reclaiming of metal polluted sites (**phytoremediation**) is gaining considerable importance, since phytoremediation is considered to be "*less invasive, more cost-effective and restorative of soil structure and functions compared to civil-engineering methods*" (Kidd *et al.* 2009).

The term phytoremediation covers a number of phytotechnologies with different characteristics (Prasad 2004, Kidd *et al.* 2009; and references therein):

- **Phytostabilization**: in situ inactivation of metals using a combination of plants and soil amendments.
- **Phytoextraction**: use of plants to absorb metals from the soil into plant roots and in some cases, translocation to above-ground plant parts.
- **Phytovolatilization**: absorption and transformation of metals into non-toxic volatile forms by plants (applied specifically to Hg and Se), and
- **Rhizofiltration**: use of plant roots to absorb, concentrate and/or precipitate heavy metals from aqueous solutions.

Considering heavy metals, all these phytotechnologies are based on the occurrence of plant species (or populations within certain species) which are tolerant

BOX 1.1 What are heavy metals? Why are they toxic? Micronutrients?

'Heavy metals' is an artificial category in which metals that have a high affinity for organic molecules are grouped. This affinity has determined that in the evolution of life some of them have acquired an essential role in animal and plant metabolism (see table A for key elements in plants) but always in low concentrations. This subgroup of heavy metals are thus considered 'micronutrients' (Epstein and Bloom 2005).

However, when a heavy metal, still being an essential element, exceeds a certain 'threshold of safety' inside the organism (see Fig. A, lower), it causes toxic effects that may result in reduction of growth, decrease of reproductive performance ('fitness') or even the death of the organism. In the case of essential heavy metals, the organism may suffer negative effects if minimal concentrations are not reached (Fig. A, upper).

The toxicity mechanisms involve one or more of the following (Hall 2002): i) Displacement of essential metal ions from biomolecules, ii) Blocking of essential functional groups of biomolecules and modification of its active conformation, iii) Disruption of the integrity of biomolecules, iv) Modification of other biologically active agent, v) Stimulation of the formation of free radicals and reactive oxygen species (ROS).

Table A: heavy metals as micronutrients

Function	Elements
Photosynthesis	Cu, Fe, Mn
Detoxification of ROS	Cu, Fe, Mn, Zn
Growth regulation	Fe, Mn
DNA transcription	Zn
Nitrogen metabolism	Co, Ni, Mo

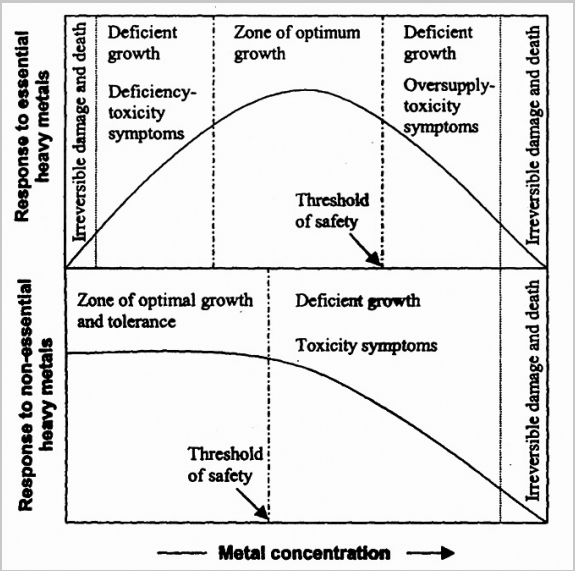

Fig. A: growth responses of plants to increasing concentrations of metals. From Shaw *et al.* (2004).

Although there are different definitions for heavy metals (Passow *et al.* 1961, Tiller 1989, Borovik 1990, Alloway 1995), we will follow Kidd *et al.* (2009) and will use interchangeably the terms 'heavy metal' or 'trace metal' throughout this document to refer to "*elements that occur in natural and perturbed environments in small amounts, and that, when present in sufficient bioavailable concentrations, are toxic to living organisms*".

BOX 1.2 Heavy metals in soils: sources, mobility and bioavailability.

The contents of heavy metals in undisturbed soils is mainly determined by the kind of parent material or bedrock and the processes of soil formation from it (Kidd *et al.* 2009, Greger 2004), together with processes of transport and deposition (atmospheric transport, …). Thus, although the mean background levels (amounts of heavy metals in soil not disturbed by human activity) are low, there are places where they may reach high concentrations (Table B).

As well as natural factors, human activities during recent millenia, and especially in the last century, have released an increasing amount of heavy metals into terrestrial and marine ecosystems. For some of these elements, the human effect has widely exceeded the natural sources. (Table B).

Element	Lithosphere	Serpentine	Calamine	Human/natural ratio of emissions
Cd	0.5		8	18.97
Co		180		
Cr		4384		
Cu	24	150	11	13.63
Hg	0.243			0.44
Ni	75	1574		3.46
Pb	31.5	21	3996	345.83
Zn	70	88	3403	23.46

Table B: Mean trace metal contents ($mg.kg^{-1}$) in Lithosphere and in serpentine and calamine soils. Ratio of human/natural emissions of metals to the Atmosphere. Modified from Friedland (1990).

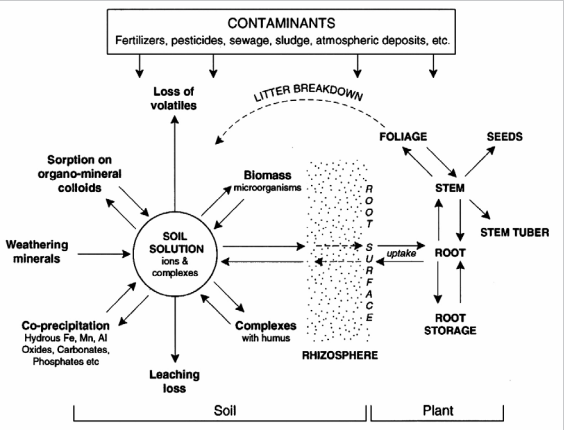

Fig. B: Plant-soil system. The most important factors related to availability and movement of metals are indicated. From Alloway (1995).

Once in soils, the mobility (retention/leachability) and bio-availability of metals is controlled by different soil properties (mainly pH, cationic exchange capacity, clay and organic matter content,) (Alloway 1995, Figure B). For most trace metals, which in soils are in the form of cations (positive charged ions), the lower clay and/or organic matter content and pH, the higher bio-availability.
In the fraction of soil surrounding plant roots (called rhizosphere) biological processes are predominant, and the collaboration of plants with different microorganisms (fungi and bacteria) may modify the metal bio-availability (Kidd *et al.* 2009).

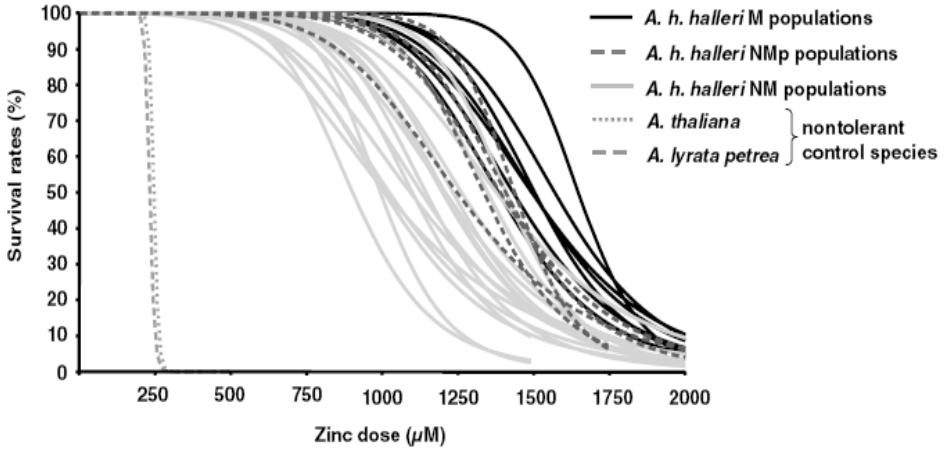

Figure 1.1: Response (measured as survival rates) of different populations of *Arabidopsis halleri*, *A. thaliana* and *A. lyrata* to increasing Zn doses. *A. halleri* populations are tolerant to Zn, whereas the other two *Arabidopsis* species are not. From Pauwels *et al.* (2006).

to heavy metals. That is, they have the capacity to colonize, survive and reproduce in a metal-polluted soil, toxic for most other plants (Antonovics *et al.* 1971, Macnair 1993).

Therefore, the recent interest in developing new lines for phytoremediation has added to the existing body of research on the mechanisms underlying heavy metal tolerance. It is desirable to identify tolerant plant species which also possess other interesting agronomic traits that make phytoremediation feasible (high biomass production, dense root and shoot systems, and even, in arid regions, adaptation to water stress; Frérot *et al.* 2006) or to dissect the genes responsive to tolerance and then transfer them (through biotechnology) into non-tolerant species with those interesting agronomic traits (Pauwels *et al.* 2008b).

The scientific approach to the phenomenon of heavy-metal tolerance has gone in two main directions: the knowledge of physiological processes and the knowledge of genecological and/or evolutive aspects.

1.1 The measurement and determination of tolerance

According to the definition we presented in previous paragraphs, tolerance to heavy metals is a complex trait defined by the interaction of genotypes and environment (Macnair 1993): different genotypes have a different response to increasing levels of metals (Fig. 1.1).

Although any species (or population) growing on a metalliferous substrate is considered as tolerant, an experimental demonstration of this tolerance (plants with different genotypes growing in a controlled environment with metals) is needed. The ideal test of tolerance should measure the effects of metal on fitness or

final yield on any species. Measuring this is difficult and laborious, so a growth parameter which is assumed to be correlated with fitness has to be used (Macnair 1993). The first and most commonly used parameter to characterise tolerance to heavy metals is the tolerance index (TI), which is calculated as:

TI = *Response to metal treatment / Response under control conditions*

Although the TI has received some criticism (Macnair 1990, Macnair 1993) it is still frequently employed, since it allows elimination of most of the variation in plant responses unrelated to metal treatment (Meyer *et al.* 2010).

Usually, the response is estimated by measuring root growth in a hydroponic culture (Wilkins 1978), but other variables such as shoot growth, leaf length or biomass are also used (reviewed in Köhl and Lösch 2004). As an alternative to these growth measures, several biomarkers, that is, biochemical, physiological or morphological changes owing to metal exposure have been used (plasmolysis capacity, pollen viability, seed germination, photosynthesis, respiration) and others have been tested in recent years (phytochelatin synthesis, ATP concentration, stress proteins…) (Köhl and Lösch 2004).

There are a wide variety of test designs for the determination of tolerance. Experiments can be developed sequentially (the same plant is first cultivated in control conditions and then subjected to a treatment with the metal) or in parallel (responses to control and to metal treatment are measured at the same time in different plants). It can be a single or multiple-concentration test, the latter using arithmetic or geometric series of metal concentrations (Schat and Ten Bookum 1992). Its duration may vary from around 2 days (short-term root elongation tests) to several weeks (long-term growth tests). In addition, the plants can be cultivated in hydroponic systems (with good reproducibility of nutrient and metal contents, accessibility to roots, easy harvesting of plants) or in solid media (whose cultivation conditions show a close resemblance to those in the field).

Given this wide diversity of approaches, as Macnair (1993) stated, "*the 'correct' test to use must be determined by the judgement of worker, based on experience of the species and metal under study*".

1.2 The physiology of tolerance

In one of the basic papers in this field, Baker (1981) ranked tolerant plants in three categories (accumulator, indicator and excluder) according to their patterns of response to increasing quantities of heavy metals in soils (Fig. 1.2).

Thus, an excluder shows low shoot levels of metals over a wide range of external concentrations up to a critical soil value above which the mechanism breaks down and unrestricted transport results. In contrast, accumulators concentrate metals in aerial plant parts from low or high soil levels, whereas 'indicators' show a proportional relationship between metal levels in

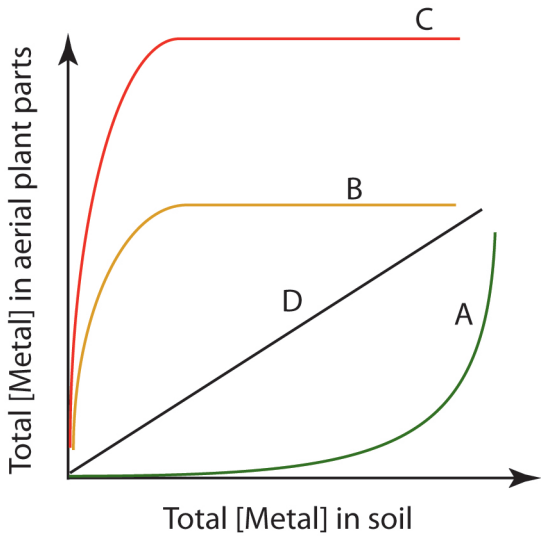

Figure 1.2: Classification of plant species on the basis of metal uptake. **A**: excluder. **B**: accumulator. **C**: hyperaccumulator. **D**: 'indicator'. Modified from Baker (1981) and Greger (2004).

the soil and accumulation in aerial plant parts. An additional category comprises the hyperaccumulators: plants that behave similarly to accumulators, but with a higher accumulation factor.

Several processes and mechanisms, both at the cellular and at whole organism level or even involving microorganisms from the rhizosphere, underlie these three general patterns.

At the cellular level, Hall (2002) cites the main mechanisms of tolerance (summarized in Fig. 1.3). Rather than developing proteins that can resist heavy metal effects, these mechanisms are aimed at preventing the build-up of toxic concentrations at the cytosol:

First, the role of rhizosphere organisms in tolerance has been demonstrated (reviewed in Kidd *et al.* 2009). Ectomycorrhizal fungi (which are exclusive to woody plants) develop a hyphae sheath that envelopes the root surface (and actually enters the intercellular spaces of the root cortex) and can reduce the inflow of metals to the plant host (point 1, Fig. 1.3). Moreover, the rhizosphere bacteria (mainly Plant Growth Promoting Rhizobacteria-PGPR) also provoke an increasing tolerance, given their role in the change in availability of metals in the rhizosphere and, especially, by secreting Plant Growth Promoting substances (referred as PGPs in point 2, Fig. 1.3).

Turning to the plant's own mechanisms, some of them act at the extracellular level. We refer to the binding of metals to the cell wall and, especially, immobilisation of metals outside the plant by binding to metal-chelating molecules exuded by roots (point 3, Fig. 1.3). E.g. *Thlaspi arvense* exposed to Ni increased the exudation of citrate and histidine by roots. These molecules form chelates with Ni and prevent its absorption by roots (Salt *et al.* 2000).

Other strategies to control the internal concentration of metals rely on the plasma membrane, mainly through reduction of the influx across the membrane by the modification of metal transporters (point 4, Fig. 1.3) e.g. arsenate tolerance in *Holcus lanatus* is related to the presence of an altered phosphate-arsenate uptake system (Meharg and Macnair 1990). The active efflux of metals through the plasma membrane to the apoplast has been proposed as another as another process involved in tolerance (point 5, Fig. 1.3). This

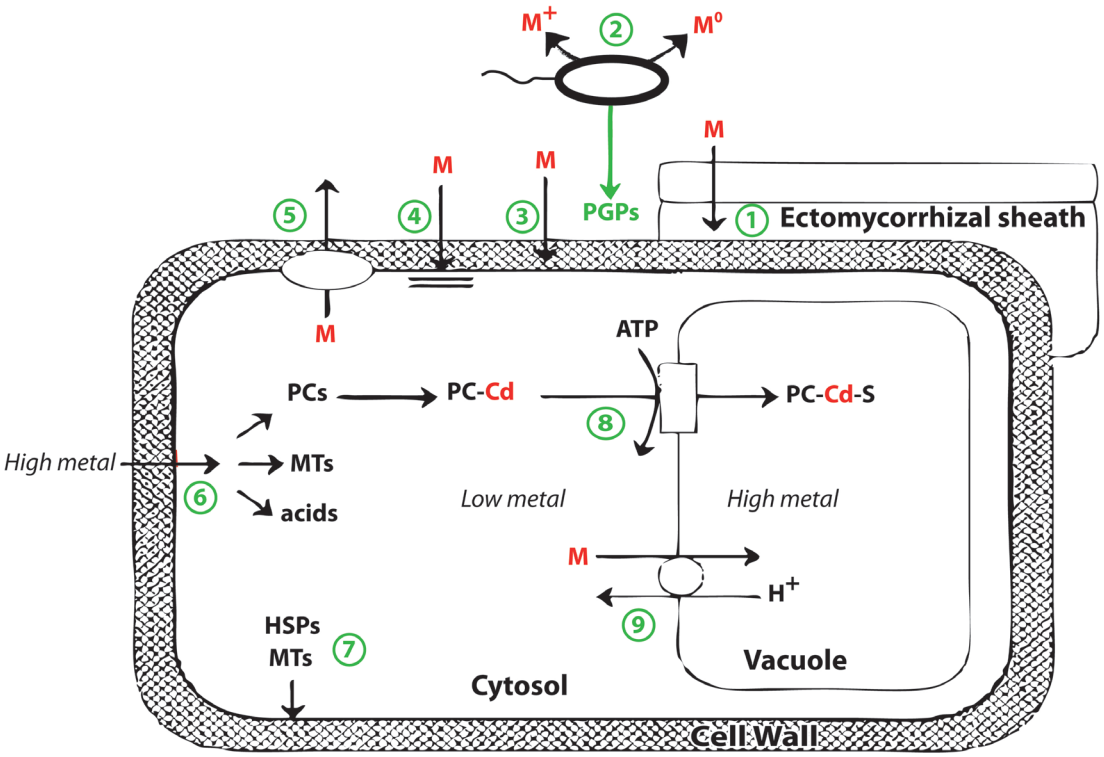

Figure 1.3: Summary of cellular mechanisms of metal tolerance in plants. **M**: metal atoms. **PCs**: phytochelatins. **MTs**: metallothioneins. **HSPs**: heat-shock proteins. **PGPs**: plant growth promoting substances. Bacteria are conventionally represented with a flagellum without any speculation on their biological status. Modified from Hall (2002).

mechanism is common in bacteria but with little direct evidence in plants (Hall 2002). For instance, metal transporter AtHMA4 has been described in *Arabidopsis thaliana*. This transporter may play a role in the translocation of Zn from root to shoot and it may be also involved in tolerance to Zn and Cd (Mills *et al.* 2005).

The remaining mechanisms are fully intracellular. They include, on one side. the chelation of metals by different organic ligands: aminoacids, organic acids and the cysteine-rich polypeptides Metallothioneins (MTs) and Phytochelatins (PCs) (point 6, Fig. 1.3). The formed chelates can then be removed from the cytosol by efflux outside the cell (e.g. complexation of Ni with histidine and transport to shoots) or pumped to the vacuole. This is the final step in the detoxification of Cd by PCs (point 8, Fig. 3).

However, free metals can be also sequestered in the vacuoles by different metal transporters in the tonoplast (point 9, Fig. 1.3). For instance, Zn tolerance in *Silene vulgaris* is mediated by theses uptake systems (Chardonnens *et al.* 1999).

The last mechanism of tolerance

to metals implies the repair and protection of plasma membranes under stressful conditions (point 7, Fig. 1.3). Heat Shock Proteins (HSPs) are the molecules mainly involved in this process, acting as chaperones in normal protein folding and assembly, as well as being involved in the protection and repair of proteins under metal stress.

Some of the mechanisms cited above are specific to the response against a particular metal, e.g. Cd detoxification by PCs; although the response to other trace metals may involve several mechanisms within the same cell or cells belonging to different tissues or organs. In this way, the redistribution of metals within the plant may play an important role in tolerance, depending on the general pattern of response (*sensu* Baker 1981).

Thus, in excluder species mechanisms to reduce the absorption of metals were predominant, and intracellular detoxification processes will occur mostly at the level of the roots. In contrast, in metal accumulators the main strategy is the transportation of metals to the aboveground plant parts and their accumulation in non-sensitive places. This mechanism is especially important in the extreme case of metal hyperaccumulating plants. For instance in *Stackhousia tryonii* (Ni hyperaccumulator) and *Thlaspi praecox* (Cd / Zn hyperaccumulator) metals are actively transported to aerial parts and accumulated preferentially in the vascular and epidermal leaf tissue, away from the photosynthetically active leaf tissues (Bhatia *et al.* 2004, Vogel-Mikuš *et al.* 2008, respectively).

1.3 Population genetics and evolution of tolerance

The study of population genetics has served to unveil the genetic bases of tolerance to heavy metals. In addition, metal-contaminated sites, either natural outcrops or mining deposits, may be considered as ecological islands which provide the opportunity to investigate the establishment and differentiation of plant populations under severe selection pressure (Lefèbvre and Vernet 1990).

Based on their presence or absence in metalliferous areas, plant species have been classified into three categories (Fig. 1.4). These categories are often related to the occurrence of genotypes tolerant to metals at the species level (Pollard *et al.* 2002):

- **Strict** (or obligate) **non-metallophytes** (species A in Fig. 1.4). Species restricted to soils with low metal concentrations, which is the case for great majority of plants.
- **Strict** (or obligate) **metallophytes**, also called eumetallophytes (species D in Fig. 1.4). These are taxa which grow exclusively on metalliferous soils. All the members of such taxa which have a tolerance to heavy metals are included Some species in this group are endemic to mine spoils or to serpentine soils, such as *Viola calaminaria* and *Alyssum serpyllifolium*, respectively.
- **Facultative metallophytes**, also called **pseudometallophytes** (species B and C in Fig. 1.4). Species with populations grow-

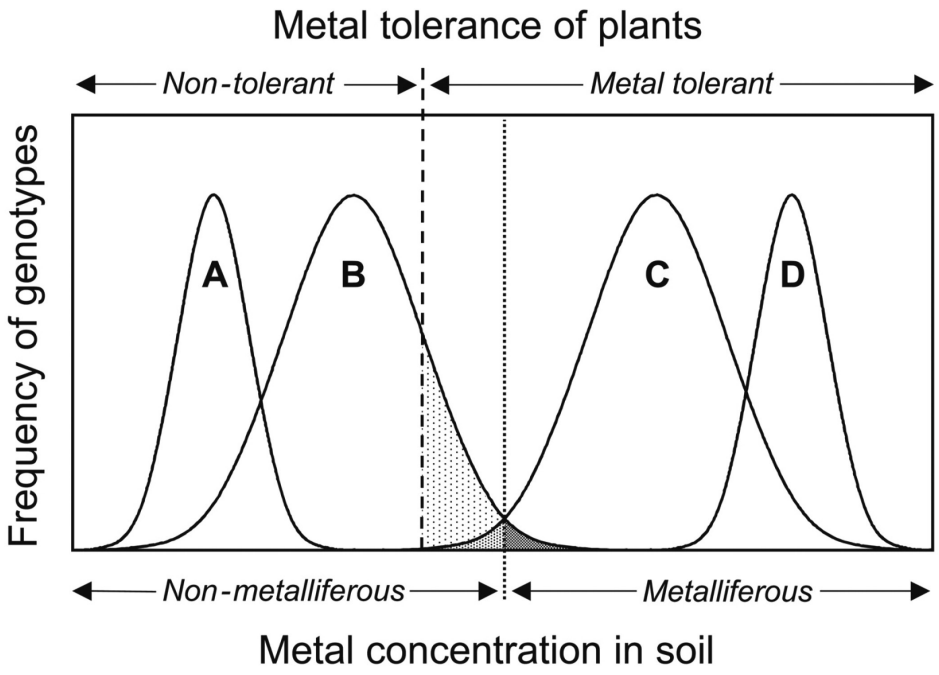

Figure 1.4: Patterns of plant metal tolerance (upper x-axis) and frequency distribution of plant genotypes on metal-containing soils exemplified for four contrasting species. **A**: strict non-metallophyte. **D**: obligate eumetallophyte. **B**: pseudometallophyte with ecotypic tolerance. **C**: pseudometallophyte with species-wide tolerance. Note that in some cases metal-tolerant genotypes are growing on non-metalliferous soils (shaded area). From Pollard *et al.* (2002).

ing on metalliferous and non-metalliferous soils belong to this group. Within the pseudometallophytes we can distinguish two sub-types: on one side, those species with **constitutive** (or **species-wide**) **tolerance**, that is, all genotypes are metal tolerant, even in those populations growing on soils with low metal contents (species C on Fig. 1.4); and on the other side, those species with **metal-tolerant races** or **ecotypes** that have evolved during the colonisation of metalliferous areas by populations where tolerant genotypes were at low frequencies (species B on Fig. 1.4) (Pollard *et al.* 2002).

To complete this relatively simple scheme, we add that several authors have stressed that plants which are, irrespective of their tolerance to metals, pre-adapted to any of the harsh edaphic conditions of metalliferous soils (nutrient shortage or imbalance, drought, high insolation, etc.) could colonize these soils more readily (Brady *et al.* 2005; and references therein), so, as Wu (1990) outlined, it is possible to colonize metalliferous soils without the evolution of tolerance.

In addition, it is important to consider the 'cost of tolerance', that is, the fact that metallicolous plants are at a

disadvantage in non-metalliferous soils. This physiological cost may explain the general absence of metallophytes in non-metalliferous substrates. Recent findings indicate that the cost of tolerance is not an effect of the tolerance genes, but is due to adaptation to the previously mentioned conditions or even to higher susceptibility to herbivores; it is therefore more precise to talk about the 'cost of adaptation to metalliferous environment' (Macnair *et al.* 2000, Dechamps *et al.* 2008 and references therein).

Given the within-species differences in tolerance to metals found in pseudometallophytes, these kinds of plants allow us to investigate the genetics and physiology of the differences between the tolerant and non-tolerant plants in a way that is generally not possible with the edaphic endemics (Macnair 1993). In addition, neighbouring metallicolous and non-metallicolous populations of pseudometallophytes are highly relevant models for the study of local adaptation in plants (Linhart and Grant 1996). Other interesting properties of pseudometallophytes are their high capacity to adapt to a wide range of adverse soil conditions, together with their usually higher bio-mass production and their good competitiveness in soils with moderate metal toxicity; they may therefore be useful for phytoremediation technologies (Poschenrieder *et al.* 2001).

On the basis of several experimental crosses between selected lines for tolerance and sensitivity to heavy metals within pseudometallophyte species, Macnair (1993) demonstrated that tolerance is a trait controlled by a small number (usually one) of major genes with main effects. This assertion is congruent with the results obtained by Wu *et al.* (1975), who indicated that evolution of tolerance can arise in a single generation.

Moreover, minor genes, present in greater numbers and less effective than major genes, have been discovered. These minor genes would be responsible for the variation in tolerance levels observed within and among metal-tolerant populations (Macnair *et al.* 2000). In addition, minor genes are hypostatic to major genes; that is, their effect on tolerance can be only observed if major genes are present.

Another interesting finding is that the co-tolerance (that is, the fact that tolerance to one metal confers a tolerance to other metals) has not been demonstrated; this fact contradicts the possible physiological role of nonspecific systems of metal tolerance, such as PCs, MTs and organic acids (Macnair *et al.* 2000).

The ease of growing, selecting and crossing herbaceous species in the laboratory has caused a certain bias in genetic studies of tolerance to herbs (Macnair 1993). Complementary to these laboratory studies, other lines of research employed population biology and genetics as tools for identifying evolutionary and genetic factors involved in tolerance (Pauwels *et al.* 2008a). Indeed, these population-based approaches are useful to study the origin and evolution of metal tolerance in woody pseudometallophyte species, with longer life cycles.

From a evolutionary point of view, researchers tried to address three major questions: i) Does colonisation of metalliferous areas imply a reduction in genetic diversity?, ii) Do metallicolous (M) populations share a common ancestry or are they the result of local colonization events? and iii) Have soil conditions promoted a significant genetic isolation between population types?

These three questions are ultimately related to the frequency of tolerance genes and also to the physiological cost of tolerance. If tolerance to heavy metals is not common in non-metallicolous (NM) populations of a certain pseudometallophyte species, it is expected that the metallicolous (M) population will be founded by a low number of mother plants. Thus, a founder effect (which implies a reduction of genetic diversity in M population and also a significante genetic differentiation between M and NM populations) may occur (Lefèbvre and Vernet 1990).

The different selection forces in metalliferous and non-metalliferous soils contribute to maintain the genetic differentiation between M and NM populations (Linhart and Grant 1996), whereas the gene flow between neighbouring M and NM populations may contribute towards homogenizing them. The differentiation between edaphic types can be promoted by the onset of barriers for gene flow between M and NM populations preventing the dilution of metal tolerance by pollen coming from non-tolerant populations (Lefèbvre and Vernet 1990). Some of these processes include increasing self-fertility in M populations, divergence in flowering time between M and NM populations, pollen-stigma incompatibiliy or hybrid sterility (Lefèbvre and Vernet 1990, Vekemans and Lefèbvre 1997).

In order to summarize the research carried out on the influence of the colonisation of metalliferous areas on the genetic structure of pseudometallophyte species, we have elaborated a table (Table 1.1), in which we present a review of papers on this topic arranged according to their year of publication. This table shows a progressive change in the molecular markers used in genetic studies (from isozymes and allozymes to DNA based markers), and also an increase in the number of populations and the geographic range considered.

These transitions are caused by the greater information provided by DNA compared to allozymes (markers very prone to homoplasy) and mainly for the need to separate the historical processes (phylogeography) from the selective processes really involved in the colonisation of M areas. As Staton *et al.* (2001) underline, the inference of the phylogeography of a species makes possible a better understanding of the effect of metal pollution on the genetic structure of populations, avoiding spurious correlations resulting from historical or demographic processes.

Among the DNA-based markers, those obtained from the chloroplast genomes (either chloroplast microsatellites –cpSSR- or chloroplast PCR-RFLP) are of special interest when studying colonisation patterns, and thus they have been widely used to infer the history of plant popula-

Table 1.1: Summary of the effects of the colonisation of metalliferous areas on the genetic diversity and/or the population genetic structure of different pseudometallophyte species.

Reference	Species	Markers	N of Pops			Colonisation of M areas		
			NM	M (s)	M (h)	Reduction Diversity	Genetic differentiation	Origin
Wu *et al.* (1975)	*Agrostis stolonifera*	Isozymes	2		9	No	n.e.	n.e.
Ducousso *et al.* (1990)	*Arrhenatherum elatius*	Allozymes	3		3	No	n.e.	n.e.
Westerbergh and Saura (1992)	*Silene dioica*	Isozymes	9	8		No	No	Multiple
Bush and Barret (1993)	*Deschampsia cespitosa*	Isozymes	8		10	Yes	Yes	Multiple
Vekemans and Lefèbvre (1997)	*Armeria maritima*	Allozymes	9	1	8	Yes	No	Multiple
Lehmann (1997)	*Calamagrostis epigejos*	Isozymes	2		2	No	n.e.	n.e.
Koch *et al.* (1998)	*Thlaspi caerulescens*	Isozymes	13		15	No	No	Multiple
Nordal *et al.* (1999)	*Lychnis alpina*	Isozymes	1		2	Yes	n.e.	Multiple
Mengoni *et al.* (2000)	*Silene paradoxa*	RAPD	1	5	2	No	Yes (mine vs. serpentines)	Multiple
Mengoni *et al.* (2001)†	*S. paradoxa*	cpSSR	1	5	2	Yes (mine pops)	No	Multiple
Nkongolo *et al.* (2001)	*D. cespitosa*	RAPD	2		7	n.e.	No	Multiple

n.e.: not estimated; † this paper analysed the same populations as Mengoni *et al.* (2000)

Table 1.1 (continued)

Reference	Species	Markers	N of Pops			Colonisation of M areas		
			NM	M (s)	M (h)	Reduction Diversity	Genetic differentiation	Origin
Nyberg Berglund and Westerbergh (2001)	*Cerastium alpinum*	Isozymes	19	12		No	No	Multiple
Dubois *et al.* (2003)	*T. caerulescens*	Allozymes	7		7	No	Yes (in one region)	n.e.
Pauwels *et al.* (2005)	*Arabidopsis halleri*	(cpDNA) PCR-RFLP	14		14	No	No	Multiple
Mengoni *et al.* (2006)	*Onosma echioides*	AFLP	3	5		No	No	n.e.
Baumbach and Hellwig (2007)	*A. maritima*	AFLP	12		10	No	No	Multiple
Deng *et al.* (2007)	*Sedum alfredii*	RAPD	2		5	Yes	Yes	n.e.
Jiménez-Ambriz *et al.* (2007)	*T. caerulescens*	nuSSR	3		3	No	No	n.e.
Pauwels *et al.* (2008)‡	*A. halleri*	(cpDNA) PCR-RFLP	50		14	No	No	Multiple

n.e.: not estimated; ‡ this paper used the same populations as Pauwels *et al.* (2005), as well as additional NM populations.

tions (Petit *et al.* 2003, Magri *et al.* 2007). CpDNA is generally maternally inherited in angiosperms, its dispersion is therefore carried out through seeds only. Thus, they are not influenced by pollen flow among M and NM populations.

In addition, the effective population size for haploid cpDNA is smaller than diploid nuclear genes, so the differentiation due to genetic drift may be stronger (Comes and Kadereit 1998) and phenomena like genetic bottlenecks may be more easily detected (Echt *et al.* 1998). For instance, cpSSR markers detected a reduction in genetic diversity within copper-mine populations of *Silene paradoxa* where RAPD markers failed (Mengoni *et al.* 2001).

Moreover, chloroplast microsatellites are considered to be neutral markers, that is, all their alleles (variants) have equal effects on the individual carrying them. Thus, cpSSRs provide a selection-independent framework which allows us to separate the effects of phylogeography from the effects of metal pollution.

As regards genetic diversity, a common trend across species is not found. In general, a founder effect has been detected in studies with populations on mining areas. This points to the effect of time together with the fact that most of the presented papers rely on markers related to nuclear DNA. Whereas the populations on mine tailings have supposedly been founded recently, the origin of populations on serpentine outcrops is generally older. A longer time implies a greater pollen flow from neighbouring non-metallicolous populations that could have increased genetic diversity and masked the putative founder effect. This is reflected by the aforementioned papers by Mengoni *et al.* (2000) and Mengoni *et al.* (2001) on *Silene paradoxa*.

In addition, several authors proposed that the increase in genetic diversity inferred in M populations of clonal grasses (e.g. *Arrhenatherum elatius* or *Calamagrostis epigejos*) is a sum of soil heterogeinity together with low intraspecific competition in polluted deposits, which allows the coexistence of a higher number of clones than in non-metalliferous soils (Ducousso *et al.* 1990, Lehmann 1997).

Only two pseudometallophytes have been studied with chloroplast markers: *Silene paradoxa* (Mengoni *et al.* 2001) and *Arabidopsis halleri* (Pauwels *et al.* 2005, Pauwels *et al.* 2008) with constrasting results. Pauwels *et al.* (2005) proposed that the colonisation of metal-polluted environments is associated with a genetic bottleneck in species with populational tolerance (as *S. paradoxa*), whereas in species with constitutive (or "specieswide") tolerance (such as *A. halleri*) the effect of a bottleneck may not be detected.

Genetic differentiation between population types has been detected in few works, and has mainly been related to geographic distances between M and NM populations than to a true effect of metals in soil (Dubois *et al.* 2003), or to a limited sampling that has made it impossible to distinguish between phylogeographic and selective effects (Bush and Barret 1993, Deng *et al.* 2007). As a rule, then, we may

conclude that the evolution of tolerance is not hampered by the existence of gene flow among populations, possibly due to high selective pressures in metalliferous soils (Vekemans and Lefèbvre 1997). This is clearly congruent with the fact that M populations have multiple origins, that is, they have originated locally from NM populations that colonised metalliferous areas. In addition, M populations from a certain area are more genetically similar to neighbouring NM populations than to distant M ones.

Given the fact that variations in tolerance and accumulation capacity are genetically controlled, metallicolous populations with independent origins might show different patterns of response to heavy metals, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Silene armeria* (Llugany *et al.* 2003), *Cerastium alpinum* (Nyberg Berglund *et al.* 2003) and *Thlaspi caerulescens* (Assunção *et al.* 2003).

Over the last two decades, the field of population genomics has developed with the aim of identifying loci causing adaptive differences in natural populations. Population genomics is based on the use of genome scans; that is, the screening of genome-wide patterns of DNA polymorphism to detect 'outlier loci' which are subjected to positive directional selection (Storz 2005, and references therein).

Based on Amplified Fragment Length Polymorphism (AFLP, Vos *et al.* 1995), a kind of DNA-marker which can be applied to any organism without previous knowledge of sequences, the genome scans have been applied to non-model organisms (Bonin *et al.* 2007). In the case of plants, genome scans with AFLP have been used for taxonomic purposes (Scotii-Saintagne *et al.* 2004, Savolainen *et al.* 2006), or to detect loci potentially involved in adaptation to different ecological conditions (Namroud *et al.* 2008, Parisod and Christin 2008, Poncet *et al.* 2010). Moreover, genome scans may play a promising role in the studies of adaptation and evolution of tolerance to heavy metals; for instance, Meyer *et al.* (2009) have inferred loci putatively involved in tolerance to heavy metals in M and NM populations of the pseudometallophyte *Arabidopsis halleri*.

1.4 *Cistus ladanifer* L., an interesting pseudometallophyte

The gum rockrose ('esteva' in Galician-Portuguese or 'jara pringosa' in Castillian-Spanish) (*Cistus ladanifer* L.; Fam. Cistaceae) is a woody shrub from the Western Mediterranean Area (from Southern France to the North of Morocco and Algeria) (Demoly and Montserrat 1993).

1.4.1 Taxonomy, description, breeding system and seed dispersal

An adult *C. ladanifer* plant may reach a height of 2m, with dense root and shoot systems (Martín Bolaños and Guinea López 1949). It has lanceolate green leaves with a glabrous upper surface, whereas the back is covered with a white tomentum. Leaves are presented in a decussate arrangement, and welded at their base. The morphology of leaves is used

as a characteristic for the identification of its three recognized subspecies: subspp. *africanus*, *ladanifer* and *sulcatus* (Demoly and Montserrat, 1993). Subsp. *africanus* has petiolate leaves whereas those of subspp. *ladanifer* and *sulcatus* are sessile. Moreover, in subsp. *sulcatus* (originally described as *Cistus palhinhae*) the leaf surface is split by well marked veins.

Subsp. *ladanifer* is primarily distributed in the Iberian Peninsula, northern Africa and France (where it is considered an introduced species); subsp. *sulcatus* is endemic to south-western Portugal (Algarve region); and subsp. *africanus* is present in southern Spain (Cádiz, Málaga), but more commonly found in northern Africa (Demoly and Montserrat 1993). Guzmán and Vargas (2009) have dated the origin of *C. ladanifer* and divergence of its different subspecies in the Upper Pleistocene.

Its flowers are large (55 to 70 mm diameter) and solitary, with 3 sepals and 5 white petals (var. *albiflorus*) which also may have a red to maroon spot at the base (var. *maculatus*). Each flower has a sessile stigma and a great number of stamens which may produce more than 700 000 pollen grains per flower (Talavera *et al.* 1993).

Pollination is entomophyllous (mainly Diptera, Hymenoptera and Coleoptera). A gametophytic mechanism of incompatibility exists, so *Cistus ladanifer* is an obligate outcrosser (Talavera et al. 1993). This fact, together with the short distances of pollen dispersal (only a few meters) results in reduced reproductive output in isolated plants (Metcalfe and Kunin 2006).

The fruit is a loculicide capsule with 5-7 to 12 locules. A single capsule may contain around 1,000 seeds, so it is estimated that a single adult *C. ladanifer* plant may produce more than 158,000 seeds each year. Seed release starts in mid-summer and continues for 8 to 10 months (Bastida and Talavera 2002). Seed dispersal is mainly barochorous, and more than 80% of seeds fall beneath the mother-plant canopy (Bastida and Talavera 2002), however different granivorous ants of genus *Goniomma* and the red-deer (*Cervus elaphus*), which feed on seeds and fruits respectively, may play an important role in seed dispersal over longer distances (Bastida and Talavera 2002; Malo and Suárez 1998).

1.4.2 Competitive traits

Cistus ladanifer populations constitute early successional stages adapted to disturbances operating in Mediterranean ecosystems, especially fire: its seeds maintain viability for several years (3-year-old seeds have a germination rate as high as 80%; *personal observation, C. Quintela-Sabarís*) and have a physical dormancy mechanism that can be interrupted by fire, high temperatures (Pérez-García 1997), smoke and nitrogenous salts (Pérez-Fernández and Rodríguez-Echeverría 2003).

The post-fire recovery of plants is accomplished by massive seedling emergence during the first post-fire year (Ferrandis *et al.* 1999), which allows rapid regeneration of original populations (2 years after experimental burning, *C. ladanifer*

covers 40% of the original area; Calvo *et al.* 2005).

Moreover, this species is well adapted to water and light stress. It develops dense shallow root systems that favour water uptake in transient wet periods (Martín Bolaños and Guinea López 1949). In addition, it is semi-deciduous, retaining small-sized potentially active leaves through the summer drought (Núñez-Olivera *et al.* 1996). Leaves are also photoprotected by the exudation of a fragrant, sticky resin (labdanum) which increases during summer (Chaves *et al.* 1993). Due to its content in flavonoids and other phenolyc compounds, the secretion of labdanum has other beneficial effects for *C. ladanifer* plants: it is an allelopathic compound that inhibits the germination of other plants (Herranz *et al.* 2006) and it provides a defence against herbivores eating *C. ladanifer* leaves through impairment of mouth skeletal muscle relaxation (Sosa *et al.* 2004).

1.4.3 Relation to soils

Cistus ladanifer is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). However, distribution models support its tolerance to calcareous soils (Gastón *et al.* 2009) and subsp. *sulcatus* is even restricted to limestone-derived soils on coasts from SW Portugal.

Its ability to develop in nutrient-poor areas may have been favoured by the establishment of symbioses with microorganisms in roots. Several bacteria strains with phosphate solubilisation or siderophore production (one of them identified as Plant Growth Promoting Rhizobacteria - PGPR) have been isolated from *C. ladanifer* roots (Ramos Solano *et al.* 2006). In addition, more than 30 fungal species have been recorded as forming symbiotic relations (ectomycorrhiza) with *C. ladanifer* in the literature (Comandini *et al.* 2006).

But most important among all these interesting features is the fact that ***C. ladanifer* is a pseudometallophyte**. Subspecies *ladanifer* and *africanus* have successfully established populations on serpentine areas and on mine tailings, where in some cases they are the dominant species.

In table 1.2 we summarize the papers reporting the occurrence of gum rockrose on metalliferous substrates, which also quantify the contents of diverse heavy metals in different plant organs collected in the field. In some of these papers (Alvarenga *et al.* 2004, Pratas *et al.* 2005, Murciego Murciego *et al.* 2006, de la Fuente *et al.* 2010), *C. ladanifer* has been described as an indicator, or even an accumulator, of As, Sb and Zn, and Mn, Sb and W, respectively.

These references provide useful information about gum rockrose and metals. However, all are local or regional-based reports, using different methodologies for the sampling and quantification of metals, so it is difficult to make comparisons between them.

To our knowledge, only three papers assess the tolerance of *C. ladanifer* to

Table 1.2: Heavy metal contents (as $mg.kg^{-1}$) quantified in different organs of field-collected *Cistus ladanifer* plants. This table includes the bibliographic reference (in alphabetical order), the area of study and the analysed plant organs. Data indicate mean values, except those data in **bold type**, which refer to maximum contents. Empty cells indicate elements not quantified in the study.

Reference	Area of study	Plant organs	Ag	Al	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	Se	Sn	W	Zn
Alados *et al.* (1999)	Ultramafic area in Málaga (S Spain)	leaves										10.1						
Alvarenga *et al.* (2004)	Pyrite mine tailing (Alentejo, S Portugal)	leaves							**29.7**		**1399**		**25.8**					**314**
		roots							**80.6**		**357**		**29.1**					**92.6**
Ater *et al.* (2000)	Bni Bouchra ultramafic area (N Morocco)	leaves										98.0						
Batista (2003)	Neves Corvo mining area (Alentejo, S Portugal)	leaves	**0.56**	**4315**	**48.2**		**11.9**	**10.1**	**591.5**		**2852**	**20.2**	**24.1**	**8.5**		**20.2**	**0.2**	**177**
		roots	**0.04**	**1788**	**6.7**		**1.4**	**55.0**	**176**		**1233**	**19.5**	**7.2**	**1.13**		**82.7**	**0.1**	**70.7**
Casado *et al.* (2007)	Losacio and Cogollas old mines (Zamora, N Spain)	aerial parts			**1.8**									**0.71**				
Chopin and Alloway (2007)	Mining area Iberian Pyrite Belt (SW Spain)	aerial parts			**30**				**460**				**237**					**729**
Díez Lázaro *et al.* (2006)	Ultramafic outcrops and surrounding area in Trás-os-Montes (NE of Portugal)	leaves						**128**	**15.0**		**2000**	**50.0**						**300**
		stems						**26.0**	**11.0**		**467**	**50.0**						**500**
		roots						**17.0**	**15.0**		**350**	**75.0**						**140**

Table 1.2 (continued)

Reference	Area of study	Plant organs	Ag	Al	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Sb	Se	Sn	W	Zn
Freitas *et al.* (2004a)	Abandoned mine within ophiolitic complex (Trás-os-Montes, NE of Portugal)	leaves					1.7	1.9	3.5		60.7	18.9	1.3					36.2
		fruits					0.7	0.4	5.3		14.9	10.8	0.7					25.6
		roots					6.3	40.8	19.4		100.3	99.9	4.1					56.2
Freitas *et al.* (2004b)	Abandoned Copper mine in SE Portugal	leaves	0.1		2.1				13.4			4.6	11.0					170.1
		twigs	0.2		1.8				10.0			3.7	21.4					80.9
De la Fuente *et al.* (2010)	Rio Tinto basin (SW Spain)	aerial parts			9.45				26.3		664	3.51	10.6					113
Millán *et al.* (2006)	Almadén mining area (C Spain)	aerial parts								**5.1**								
Murciego Murciego *et al.* (2007)	Sb mining areas (Extremadura, SW Spain)	leaves												96.0				
Pratas (1996)	Borralhal mine (C Portugal)	leaves			**1.36**				**17.65**				**6.32**					**144.7**
Pratas *et al.* (2005)	Two abandoned mines in C Portugal	leaves			**2.77**									**10.6**			**30.7**	
		twigs			**2.38**									**29.3**			**3.55**	
Reglero *et al.* (2008)	Old lead mining area (C Spain)	leaves			0.25	0.49			12.5				13.6		0.37			98.5
		flowers			*nd*	0.16			10.8				2.60		0.17			36.9
Santos *et al.* (2009)	Abandoned Copper mine in SE Portugal	young leaves			1.2				8.1				47.4					127.0
Soldevilla *et al.* (1992)	Rio Tinto pyrite mine complex (SW Spain)	leaves		282		1.8			42		691		8					127

nd: not detected

heavy metals, each of them using different approaches.

- Alados *et al.* (1999), using developmental stability analysis demonstrated the adaptation of *C. ladanifer* to serpentine soils in Málaga (S of Spain). These authors also introduce the hypothesis that the low Ca^{2+} requirements of this species could be an advantage in the colonisation of serpentines. Along these lines, Ater *et al.* (2000) quantified a high Mg/Ca ratio in leaves of *C. ladanifer* growing in serpentines from N of Morocco.
- Kidd *et al.* (2004) subjected plantlets of 5 populations growing on metalliferous and non-metalliferous soils from NE Portugal to experiments of tolerance to Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn in hydroponic culture. They observed population-specific patterns of tolerance and accumulation and inferred that even plants from non-metallicolous populations showed relative tolerance to metals. We analysed 4 of these populations with RAPD markers and we found similar levels of genetic diversity within metallicolous and non-metallicolous populations, in addition we found a significant differentiation between the groups of metallicolous and non-metallicolous populations. However, we were not able to determine if the origin of this structuring was related to phylogeography or to the colonisation of metalliferous areas (Quintela-Sabarís *et al.* 2005).
- Santos *et al.* (2009) sought differential activity of antioxidative enzymes in *C. ladanifer* plants from an abandoned mine area in SE Portugal, but they did not find any variations related specifically to metals.

In a highly interesting PhD Thesis, Díez-Lázaro (2008) dealt with the optimisation of the use of *Cistus ladanifer* for phytoremediation procedures. He found that the addition of fertilizers and the acidification of soil improve the growth and the extraction of Mn and Zn by C. ladanifer plants from NE Portugal. In addition, he stated that gum rockrose could be readily used for the phytoextraction of Zn in soils with low to medium contents of this metal.

Finally, the beneficial effect of this plant is underlined by Simões *et al.* (2009), who found that it can produce up to 4,600 kg of dry matter ha^{-1} $year^{-1}$ of litterfall, which improves soil quality and may promote vegetation regeneration by facilitating the invasion of more demanding species.

In summary, *C. ladanifer* possesses a series of interesting traits that make it especially useful for the recovery of degraded areas in the Mediterranean region. In addition, it is a native species to this biodiversity-rich region, and therefore its use would not produce the detrimental effects on the surrounding ecosystems produced by alien and invasive species (Méndez and Maier, 2008; and references therein).

1.5 Objectives

According to the items presented above, *Cistus ladanifer* seems to be a promising species in phytoremediation procedures in the Mediterranean region and is also an interesting model species for

the study of the process of colonisation of metalliferous areas by plants.

Within this framework, and with the aim of improving the knowledge about *Cistus ladanifer* and its relationships with metals, we have developed a series of investigations using populations sampled from nearly the entire distribution area, in order to deal with the following topics:

- In order to infer the effects of the metals on the genetics of the species, it is first of all essential to understand the interplay of processes that creates its phylogeography, or genetic landscape. Using neutral maternally-inherited markers (cpSSRs) we inferred the phylogeography of *Cistus ladanifer* (**Chapter 2**).

- Then we integrated the soil type (metalliferous or non-metalliferous) and the phylogeographic information in a population-genetics approach to the tolerance of *Cistus ladanifer* to metals: are the metallicolous populations mono- or polyphyletic? Is the colonisation of metalliferous areas accompanied by a reduction in genetic diversity? (**Chapter 3**).

- A species range is not homogeneous, but it is often subdivided in into genetic subgroups. If metallicolous populations have evolved independently within different subgroups, it is interesting to infer whether the parallel evolution resulted in similar or different strategies of tolerance (exclusion, accumulation?). We assessed this theme, within the framework provided by cpSSR, through the analysis of field-collected soils and *C. ladanifer* leaves (**Chapter 4**) and through hydroponic-based experiments of tolerance to Co, Ni and Zn (**Chapter 5**).

- As a basis for future research, the identification of markers potentially linked to tolerance to metalliferous soils is especially needed in non-model plants such as *C. ladanifer*. We address this topic applying generalized estimating equations (GEE) to AFLP markers and data of total metal contents in soils (**Chapter 6**). We also compared the information on population genetics provided by AFLP markers (from nuclear DNA) and cpSSRs.

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Chapter 2

Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region

This chapter reproduces the published paper: Quintela-Sabarís C, Vendramin GG, Castro-Fernández D, Fraga MI (2011) Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region. Plant Biology 13:391-400

Previous page: General view of the vegetation in the Despeñaperros Gorge area (Sierra Morena, Jaén province, S of Spain). Here, *Cistus ladanifer* subsp. *ladanifer* coexists with *Quercus ilex* and *Juniperus oxycedrus* plants. (Photo: C. Quintela-Sabarís)

CHAPTER 2

Chloroplast DNA phylogeography of the shrub *Cistus ladanifer* L. (Cistaceae) in the highly diverse Western Mediterranean region

Keywords: Chloroplast microsatellites; glacial refugia; Iberian Peninsula; phylogeography; population genetics; Strait of Gibraltar.

ABSTRACT

This study investigated the phylogeographic structure of *Cistus ladanifer*, in order to locate its Quaternary refugia, reconstruct its recolonisation patterns and assess the role of geographical features (mountain ranges, rivers and the Strait of Gibraltar) as barriers to its seed flow and expansion through the Western Mediterranean.

Thirty-eight populations were screened for length variation of polymorphic chloroplast simple sequence repeats (cpSSRs). Statistical analyses included estimation of haplotypic diversity, hierarchical analysis of molecular variation (AMOVA) and fixation indices. Mantel tests, SAMOVA and BARRIER analyses were applied to evaluate the geographical partitioning of genetic diversity across the entire species range.

Pollen data from bibliography were used to complement molecular inferences Chlorotype diversity within populations was similar throughout the natural range of *C. ladanifer* (mean haplotypic diversity = 0.32). High differentiation among populations was estimated (GST = 0.60).

Our data suggest that the barriers of the Strait of Gibraltar and the Betic ranges may have favoured the divergence during glacial periods of four different lineages of populations inferred with SAMOVA. The main northward colonisation of in the Iberian Peninsula occurred from refugia in southwest Iberia. This process may have been influenced by human activities (forest clearance, livestock grazing and even commerce) in the Iberian Peninsula. In contrast, populations in the Betic area have conserved a specific haplotype.

2.1 Introduction

The Iberian Peninsula is considered one of the most important Pleistocene glacial refugia in Europe. It is a large area (about 580,000 km^2) with a high degree of physiographic, climatic and geologic variability, which has favoured the occurrence of multiple isolated glacial refugia (Gómez and Lunt 2007). During the last glacial maximum, Mediterranean taxa were restricted to southern and southeast edges of the Iberian Peninsula and to large areas of North Africa (Carrión *et al.* 2003, López de Heredia *et al.* 2007). These taxa expanded from the refugia during interglacial periods. According to the 'leading edge' hypothesis, the post-glacial expansion of species range mainly involved populations from northern edges of the refugia, which spread rapidly (by means of long-distance dispersal events) into new territories and significantly precluded the northward expansion of later-arriving lineages (Hewitt 2001). This hypothesis also predicts a significant geographical separation of lineages expanding from different refugia and, because of multiple successive founder events, a northward decrease in genetic diversity.

In addition to the geographical position of the refugia, the presence of

barriers that preclude or limit expansion is another important factor that plays a role in shaping the present genetic structure of plant populations (Taberlet *et al.* 1998): there are several mountain ranges in the Iberian Peninsula with an east–west orientation. These orographic barriers have, on the one hand, enabled survival of populations by altitudinal shifts as a consequence of climatic changes (e.g., *Pinus sylvestris*; Sinclair *et al.* 1999) but, on the other hand, might represent effective barriers to gene flow and recolonisation along the north–south axis. As well as mountain ranges, the Strait of Gibraltar could have influenced the genetic structure of plant species that survived in southern Spain during the Pleistocene ice ages successive range contractions and expansions. However, the role of this strait as a biogeographic barrier differs among plant species according to Rodríguez-Sánchez *et al.* (2008). These authors observed an absence of significant differentiation among populations separated by the Strait of Gibraltar in pioneer species with high seed dispersal and establishment potential. Human activities represent an additional factor, which, in combination with geological history and climate changes, have shaped plant diversity in the Mediterranean region (Thompson 2005). Human impact through forest clearing, use of fire and grazing caused retraction of some species to isolated patches, but also created new opportunities for colonisation and the spread of other species (e.g., *Cistus ladanifer*).

Cistus ladanifer L. (gum rockrose) is a woody, semi-deciduous shrub that grows in a wide range of habitats in the Western Mediterranean (South of France, Iberian Peninsula and northern Algeria and Morocco) (Demoly and Montserrat 1993), where it constitutes a major component of the landscape. Populations of this species represent early successional stages adapted to disturbances in Mediterranean ecosystems, particularly fire (Bastida and Talavera 2002). *C. ladanifer* is a major element of the dehesas and montados, forests of *Quercus ilex* and *Q. suber* in southwest Spain and south Portugal, partially cleared to enable extensive livestock grazing. Its role as a coloniser of disturbed areas and its distribution on both sides of the Strait of Gibraltar and throughout the Iberian Peninsula, make this an interesting species for phylogeographic and local differentiation studies. The flowers produce high quantities of pollen (mean 631,509 grains / flower; Talavera *et al.* 1993), which is mainly dispersed over short distances (Metcalfe and Kunin 2006). However, only well conserved *C. ladanifer* pollen grains can be easily distinguished from those of other *Cistus* species, so paleoenvironmental reconstructions generally use the '*Cistus* type' category, where pollen from different *Cistus* species and even other Cistaceae genera (such as *Helianthemum* or *Xolantha*) are considered together. This fact makes it difficult to reconstruct the expansion of *C. ladanifer* based solely on pollen data, particularly in the Iberian Peninsula and Morocco, where 12 *Cistus* species occur, each with different ecological requirements (Demoly and Montserrat 1993, Soriano 2002).

The use of molecular markers has enabled clarification of the post-glacial migration of plant species for which there are very limited fossil pollen records, such as *Ilex aquifolium* and *Hedera* species (Grivet and Petit 2002, Rendell and Ennos 2003). Chloroplast DNA (cpDNA) is inherited through the maternal line in *C. ladanifer* (Guzmán and Vargas 2009), and thus reflects only the effect of seed flow and seed dispersal. In addition, the effective population size for haploid cpDNA is smaller than for diploid nuclear genes, so that differentiation through genetic drift may be stronger (Comes and Kadereit 1998). These characteristics justify the wide use of cpDNA to infer the history of plant populations (Petit *et al.* 2003, Magri *et al.* 2007, Petit and Vendramin 2007). We analysed chloroplast microsatellite (cpSSR) variation in *C. ladanifer* throughout its distribution range in order to: (i) locate its putative Quaternary refugia and reconstruct its recolonisation patterns in the highly heterogeneous Iberian Peninsula; and (ii) assess the role of geographic features such as mountain ranges, rivers and the Strait of Gibraltar as barriers to seed flow and expansion of the species. Whenever possible, molecular evidence is complemented with pollen data from bibliographic references.

2.2 Material and Methods

2.2.1 The Species

Cistus ladanifer is an entomophyllous, obligatory outcrossing species, with a gametophytic mechanism of self-incompatibility (Talavera *et al.* 1993). An individual plant can produce more than 100,000 small, long-lived seeds each year (Bastida and Talavera 2002), which mainly fall beneath the mother plant canopy (Malo and Suárez 1998, Bastida and Talavera 2002). The seeds have a physical dormancy mechanism that can be interrupted by fire, high temperatures (Pérez-García 1997) and smoke and nitrogenous salts (Pérez-Fernández and Rodrıíguez-Echeverría 2003). The post-fire recovery of plants in the Cistaceae is accomplished by massive seedling emergence during the first post-fire year (Ferrandis *et al.* 1999), which allows rapid regeneration of original populations (2 years after experimental burning, *C. ladanifer* covers 40% of the original area; Calvo *et al.* 2005). Although the main dispersal strategy of this species is barochory, different authors have described endozoochory by red deer (Malo and Suárez 1998) and sheep (Manzano *et al.* 2005) as other mechanisms of long-distance seed dispersal.

Three *C. ladanifer* subspecies (*africanus*, *ladanifer* and *sulcatus*) have been described based on leaf traits (Demoly and Montserrat 1993). Subspecies *ladanifer* and *africanus* are present in the Iberian Peninsula and in North Africa, whereas subspecies *sulcatus* is restricted to limestone-derived soils on the southwest Iberian coast. A recent work (Guzmán and Vargas 2009) has dated the origin of C. ladanifer and divergence of its different subspecies in the Upper Pleistocene.

2.2.2 Plant sampling

Thirty-eight *C. ladanifer* populations, covering almost the entire natural range of the species and its three subspecies, were sampled (Table 2.1). A longitudinal transect was established at each population. Ten plants separated by at least 5 m were selected along the transect and their ripe fruits were collected. Seeds were sown in Petri dishes and grown to seedling stage in the laboratory. One seedling per mother plant was selected for the subsequent analyses. Young plants were frozen in liquid nitrogen and conserved at)20 C until DNA extraction. In three populations (ESA, FVI and FVII), DNA was extracted from field-collected mature leaves from at least nine mother plants.

2.2.3 DNA extraction

DNA was extracted from 100 mg of frozen leaves with a Dneasy® Plant Mini Kit (Qiagen, Hilda, Germany), following the manufacturer's instructions. In several cases, an additional wash with 500 µl of absolute ethanol was necessary to remove secondary compounds from the DNA extracts.

2.2.4 Microsatellite analysis

In an initial screening, universal cpSSR primers ccmp1 to ccmp10 (Weising and Gardner 1999) and Fagaceae cpSSR primers cmcs 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13 and 14 (Sebastiani *et al.* 2004) were tested on a subset of 30 samples from six geographically distant populations. Only primers ccmp1, ccmp2, ccmp3, ccmp5, ccmp10 and cmcs1 yielded consistent amplifications, of which ccmp1, ccmp5, ccmp10 and cmcs1 were monomorphic. The two polymorphic cpSSRs were then used to amplify all samples of the 38 populations. Amplification reactions were performed in a total volume of 12.5 µl containing 0.5 U GoTaq®DNA Polymerase (Promega, Madison, WI, USA), under standard reaction conditions. DNA was amplified under the following thermal profile: one denaturation cycle of 4 min at 95 C, followed by 25 cycles each consisting of 95 C for 30 s, 50 C for 30 s, 72 C for 30 s, with a final extension step at 72 C for 8 min. PCR products were loaded onto a 96 capillary automatic sequencer MegaBACE 1000 (GE Healthcare, Uppsala, Sweden). MegaBACE ET400 (GE Healthcare) was used as size standard. Fragment lengths were determined with the MegaBACE Fragment Profiler software, version 1.2 (GE Healthcare).

Table 2.1: Populations included in this work. The country of origin is shown next to population name. *FR*: France; *MO*: Morocco; *PT*: Portugal; *SP*: Spain. *Subsp*: *C. ladanifer* subspecies. *afr*: subsp. *africanus*. *lad*: subsp. *ladanifer*. *sul*: subsp. *sulcatus*. Latitude and longitude are shown as decimal degrees; *N.S.*: number of plants surveyed in each population. The last three columns indicate different indices of within-population haplotypic diversity: N_e: effective number of haplotypes; H_E: Nei's (1987) haplotypic diversity; D^2_{SH}: average genetic distances between individuals (Vendramin *et al.* 1998); *Mean*: mean ± standard deviation values.

Population/Country	Code	Subsp	Lat	Long	N.S.	N_e	H_E	D^2_{SH}
Ketama /MO	MKE	afr	34.95º N	4.64º W	10	1.52	0.38	0.22
Bni Hadifa /MO	MBH	afr	35.02º N	4.17º W	10	1.22	0.20	0.10
Bab Tazaa /MO	MBT	lad	35.09º N	5.24º W	10	1.22	0.20	0.10
El Jebha /MO	MEJ	afr	35.18º N	4.64º W	10	1.00	0.00	0.00
Bni Bouchra/MO	MSI	afr	35.30º N	4.90º W	10	1.92	0.53	0.27
Bni Bouchra /MO	MSII	afr	35.30º N	4.89º W	12	2.77	0.70	0.58
Tanger /MO	MTA	afr	35.78º N	5.93º W	10	1.00	0.00	0.00
Almodóvar /SP	EAL	lad	36.16º N	5.65º W	10	1.00	0.00	0.00
Benalup de Sidonia /SP	EBE	afr	36.32º N	5.73º W	10	2.38	0.64	0.76
Sierra Bermeja /SP	ESB	lad	36.48º N	5.18º W	10	1.00	0.00	0.00
Sierra Palmitera /SP	ESP	lad	36.60º N	5.07º W	9	1.59	0.42	0.50
Tolox /SP	ETO	lad	36.68º N	4.93º W	10	1.22	0.20	0.10
Grazalema /SP	EGR	lad	36.78º N	5.27º W	10	1.00	0.00	0.00
Sierra de Aguas /SP	EAC	lad	36.84º N	4.79º W	10	1.00	0.00	0.00
Sierra Alhamilla /SP	ESA	lad	36.99º N	2.30º W	10	1.00	0.00	0.00
São Vicente cape /PT	PSV	sul	37.03º N	8.98º W	10	1.22	0.20	0.10
Burgau /PT	PBU	sul	37.07º N	8.78º W	9	1.53	0.39	0.19
Mazagón /SP	EMA	lad	37.15º N	6.84º W	10	2.27	0.62	1.78
Corte Figueira /PT	PCF	lad	37.39º N	8.03º W	9	1.53	0.39	0.19
Aljustrel /PT	PAL	lad	37.88º N	8.18º W	9	2.31	0.64	0.53
Cardeña /SP	ECA	lad	38.28º N	4.36º W	10	2.78	0.71	0.89
Despeñaperros /SP	EDE	lad	38.39º N	3.51º W	10	2.38	0.64	0.46
El Guijo /SP	EGJ	lad	38.52º N	4.77º W	10	1.72	0.47	0.23
La Codosera /SP	ECO	lad	39.19º N	7.08º W	10	2.00	0.56	0.28
Valdecaballeros /SP	EVC	lad	39.33º N	5.34º W	10	2.78	0.71	0.71
Martinchel /PT	PMA	lad	39.52º N	8.29º W	3	1.80	0.67	5.33
Vela /PT	PVE	lad	40.44º N	7.29º W	9	1.00	0.00	0.00
Ciudad Rodrigo /SP	ECR	lad	40.63º N	6.49º W	10	1.47	0.36	0.18
Sierra Guadarrama /SP	EGU	lad	40.68º N	4.10º W	10	1.22	0.20	0.10
Fuente Saúco /SP	EFS	lad	41.19º N	5.51º W	10	2.38	0.64	0.46
Macedo dos Cavaleiros /PT	PMC	lad	41.52º N	6.82º W	10	1.00	0.00	0.00
Ricobayo /SP	ERB	lad	41.70º N	5.81º W	10	1.92	0.53	0.27
Samil /PT	PSA	lad	41.78º N	6.75º W	7	1.00	0.00	0.00
Bragança /PT	PBR	lad	41.85º N	6.87º W	10	1.92	0.53	0.27
Monte Furado /SP	EMF	lad	42.39º N	7.20º W	10	1.00	0.00	0.00
Bárcena /SP	EBA	lad	42.57º N	6.56º W	10	1.85	0.51	0.72
La Bouverie /FR	FVI	lad	43.48º N	6.66º E	9	1.00	0.00	0.00
La Bouverie /FR	FVII	lad	43.49º N	6.66º E	9	1.00	0.00	0.00
				Mean ± SD		1.58 ± 0.59	0.32 ± 0.27	0.40 ± 0.90

2.2.5 Data analysis

Genetic diversity within populations was assessed as the effective number of haplotypes ($Ne = 1/\Sigma p_i^2$), the haplotypic diversity ($H_E = [n/(n-1)][1-\Sigma p_i^2]$, where n is the number of individuals analysed in a population and p_i is the frequency of the ith haplotype in a population; Nei 1987) and the D^2_{SH} measure, as defined by Vendramin *et al.* (1998). The latter measure takes into account the difference in number of repeats among the different cpDNA haplotypes considered. The correlation between population genetic diversity parameters and latitude was assessed using the Spearman's correlation index. Phylogenetic relationships among haplotypes were inferred with Network 4.5 (Fluxus Technology Ltd. at http://www.fluxus-engineering.com/sharenet.htm) by the median joining (MJ) method (Bandelt *et al.* 1999).

Genetic differentiation among populations was estimated by analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) with Arlequin software (version 3.11; Excoffier *et al.* 2005). The significance of the values was computed by a permutation test with 10,000 permutated matrices. The AMOVA was based on distances between cpSSR haplotypes, calculated as the sum of the squared number of repeat differences between two haplotypes: $d_{xy} = \Sigma[a_{xi} - a_{yi}]^2$ (where a_{xi} and a_{yi} are the number of repeats for the ith locus in haplotypes x and y). This gives Φ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin 1995) for population differentiation (Michalakis and Excoffier 1996).

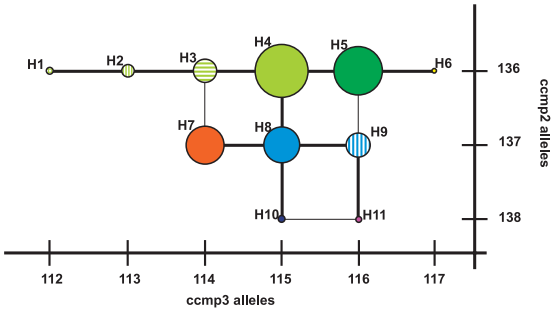

Figure 2.1: Median Joining (MJ) network for *Cistus ladanifer* cpSSR haplotypes. Axes indicate the size of ccmp2 and ccmp3 variants for each haplotype. Thin lines in each of the three loops indicate links that could be removed following the coalescence theory predictions (Crandall and Templeton 1993). Haplotypes are indicated with the same patterns as in Fig.2.2

The possible presence of geographic structure was evaluated with several tests. First, we tested for the presence of a phylogeographic structure by comparing differentiation of unordered alleles (G_{ST}) and ordered alleles (R_{ST}) with the Permut CpSSR 2.0 software (Pons and Petit 1996; http:// www.pierroton.inra.fr/genetics/labo/Software). One thousand random permutations of haplotype identities were carried out, while maintaining haplotype frequencies and the matrix of pair-wise haplotype differences as in the original study (Burban *et al.* 1999). If R_{ST}, which takes into account genetic differences between the haplotypes, is significantly higher than G_{ST}, this indicates the presence of a phylogeographical structure (Pons and Petit 1996), i.e., closely related haplotypes are more often found in the same geographical area than would be expected by chance. Second, we tested for a pattern of isolation by distance. A Mantel test with 10,000 ran-

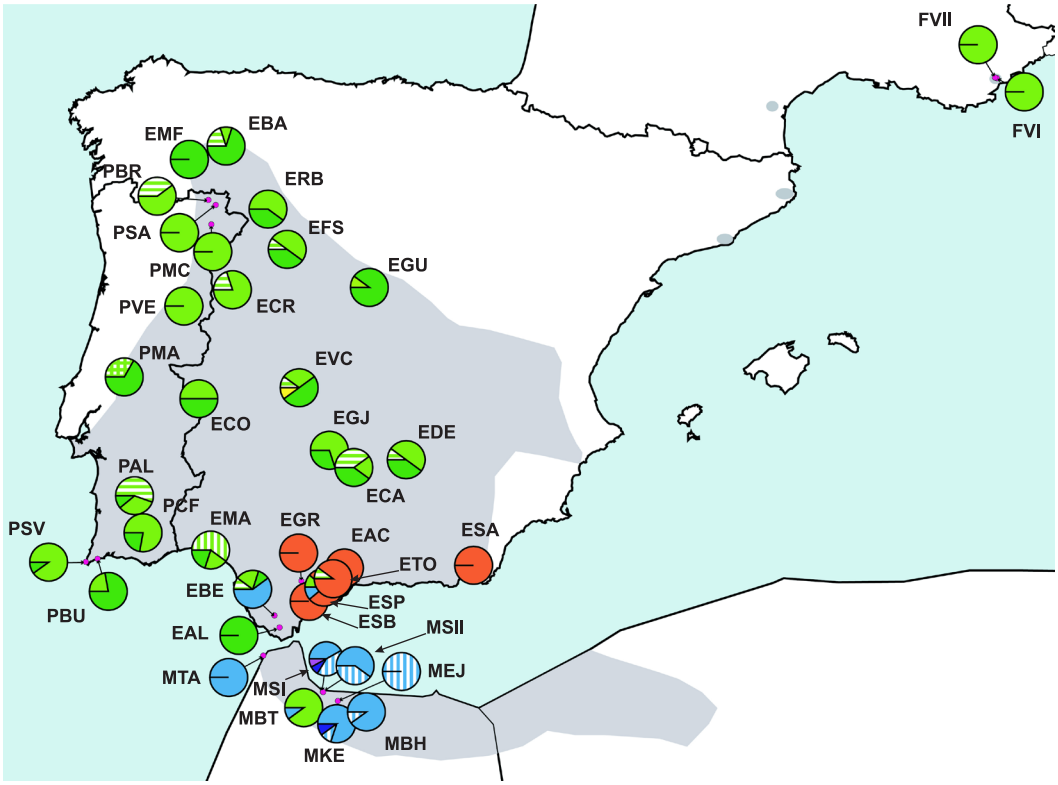

Figure 2.2: *Cistus ladanifer* cpSSR haplotype map. Grey-shaded area indicates the present natural distribution of *C. ladanifer*. The same patterns are used for haplotypes as in Fig. 2.1. In order to obtain a proper view of haplotype distribution, some radial graphs were displaced from their original position (arrows connect them with their original position). Population codes (see Table 2.1) are presented next to each radial graph.

dom permutations was performed with the matrix of pair-wise genetic differentiation between populations, $(\Phi_{ST}/(1 - \Phi_{ST}))$, and a matrix of the ln (geographic distance) with the Arlequin 3.11 software (Excoffier *et al.* 2005). Third, we used a simulated annealing procedure implemented in the spatial analysis of molecular variance (SAMOVA) algorithm (Dupanloup *et al.* 2002) to define groups of populations that are geographically homogeneous and maximally differentiated from each other. The program repeatedly seeks the composition of a user-defined number, K, of groups of geographically adjacent populations that maximises Φ_{CT}, the proportion of total genetic variance due to differences among groups of populations. The program was run for 10,000 iterations for K values from K = 2 to K = 15 from each of 100 random initial conditions. Within each of the groups defined by SAMOVA, separate AMOVA analyses were performed to partition the genetic diversity at intra- and inter-population levels. Fourth, we tested for the presence of genetic barriers among

populations using the Monmonier algorithm implemented in the BARRIER 2.2 software (Manni *et al.* 2004). Virtual points were added to the original tessellation/triangulation in order to indicate the presence of the Mediterranean Sea barrier and to enable connections to be established among populations from the South of France and central Iberian Peninsula. The distances used were pair-wise Φ_{ST} (Michalakis and Excoffier 1996). Statistical confidence for the predicted barriers

Table 2.2: Analysis of molecular variance (AMOVA) of *Cistus ladanifer* (a) considering the whole data set, (b) SAMOVA groups (K = 4), (c to e) separate analyses for three groups defined by SAMOVA analysis and (f) hierarchical geographic AMOVA comparing groups of populations to the north and south of the Strait of Gibraltar.

Source of variation	d.f.	SS	Variance components	% of total variance	P
(a) Whole data set (Φ_{ST} = 0.66)					
Among populations	37	212.70	0.57	66.35	< 0.0001
Within populations	327	94.31	0.29	33.65	
Total	364	307.01	0.86		
(b) SAMOVA results (Φ_{CT} = 0.67)					
Among SAMOVA groups	3	164.19	0.82	66.83	< 0.0001
Among pops. within SAMOVA groups	34	48.51	0.12	9.69	< 0.0001
Within populations	327	94.31	0.29	23.48	< 0.0001
Total	364	307.01	1.23		
(c) Rif group (Φ_{ST} = 0.42)					
Among populations	5	7.28	0.12	41.59	< 0.0001
Within populations	56	9.77	0.17	58.41	
Total	61	17.05	0.30		
(d) Betic group (Φ_{ST} = 0.06)					
Among populations	5	0.52	0.00	6.18	N.S.
Within populations	53	3.34	0.06	93.82	
Total	58	3.86	0.07		
(e) Western group (Φ_{ST} = 0.32)					
Among populations	24	40.71	0.15	32.18	< 0.0001
Within populations	209	65.20	0.31	67.82	
Total	233	105.91	0.46		
(f) Geographic AMOVA (Φ_{CT} = 0.25)					
Iberian Peninsula vs. N Morocco	1	35.12	0.26	25.07	0.003
Among populations within groups	36	177.58	0.48	46.97	< 0.0001
Within populations	327	94.31	0.29	27.96	< 0.0001
Total	364	307.014	1.03		

d.f. = degrees of freedom, *SS* = sum of squared deviation, *P* = level of probability of obtaining a more extreme component estimate by chance alone. *n.s.* = not significant (α = 0.05).

was obtained by resampling individuals within populations in order to obtain 100 bootstrap replicates of each genetic distance matrix. A hierarchical AMOVA was then performed in order to assess the partitioning of variance between the Iberian Peninsula and North Morocco. In addition, the genetic differences among populations were visualised through a principal coordinate analysis (PCoA) performed using GenAlEx 6.3 (Peakall and Smouse 2006) and a Euclidean genetic haploid distance similar to the genetic binary distance described by Huff *et al.* (1993).

2.3 Results

The two polymorphic microsatellites, ccmp2 (three size variants) and ccmp3 (six size variants), were combined into 11 different haplotypes among the 365 individuals analysed (Fig. 2.1). The number of haplotypes per population ranged from 1 to 4, while the mean effective number of haplotypes for the 38 populations was 1.58 (with a range of variation between 1.00 and 2.78). In addition, the average H_E and D^2_{SH} values were 0.32 (0.00–0.71) and 0.40 (0.00–5.33), respectively (Table 2.1). None of the three diversity indices used was correlated with latitude: we can find depauperated populations (with diversity values = 0.00) and also diverse populations throughout the range of *C. ladanifer*. Three haplotypes (H1, H6 and H11) were singletons, while H2 was unique to one population in southern Spain (EMA). More than 30% of the plants shared the same haplotype (H4). Two haplotypes (H4 and H8) were present on both sides of the Strait of Gibraltar, whereas haplotype H7 only appeared in populations from southeast Spain (Fig. 2.2). Median-joining analysis resulted in a complex haplotype network, with three loops (Fig. 2.1). Haplotype H8 occupies a central place within the three loops and connects the common haplotypes, H3 and H7.

Although a phylogeographic structure was not detected by the permutation analysis (G_{ST} not significantly different from R_{ST}), a strong genetic structure was observed in *C. ladanifer* populations, with high values of both G_{ST} (0.60 ± 0.06) and R_{ST} (0.57 ± 0.12). The AMOVA analysis also revealed a high and significant value for inter-population differentiation (Φ_{ST} = 0.66) (Table 2). The isolation by distance test showed that among-population differentiation increased significantly with the ln (geographical distance) (Mantel test; $P < 0.05$), although the regression accounted for only a very low proportion of the total variance ($r^2 = 0.013$). The SAMOVA analyses indicated distinct groups of genetically defined geographic areas; when K = 2, a group that comprised populations from the Betic area (EAC, EGR, ESA, ESB, ESP, ETO) was separated from the rest of the populations ($\Phi_{CT} = 0.61$). In analyses where K = 3, an additional partition was identified that subdivided the second group into two areas: one comprising populations from the South of France, the whole of the Iberian Peninsula (except the Betic area) and a population from North Morocco; the other comprising populations of *C. ladanifer* subsp. *africanus* from the Rif area ($\Phi_{CT} = 0.64$). When K = 4, a

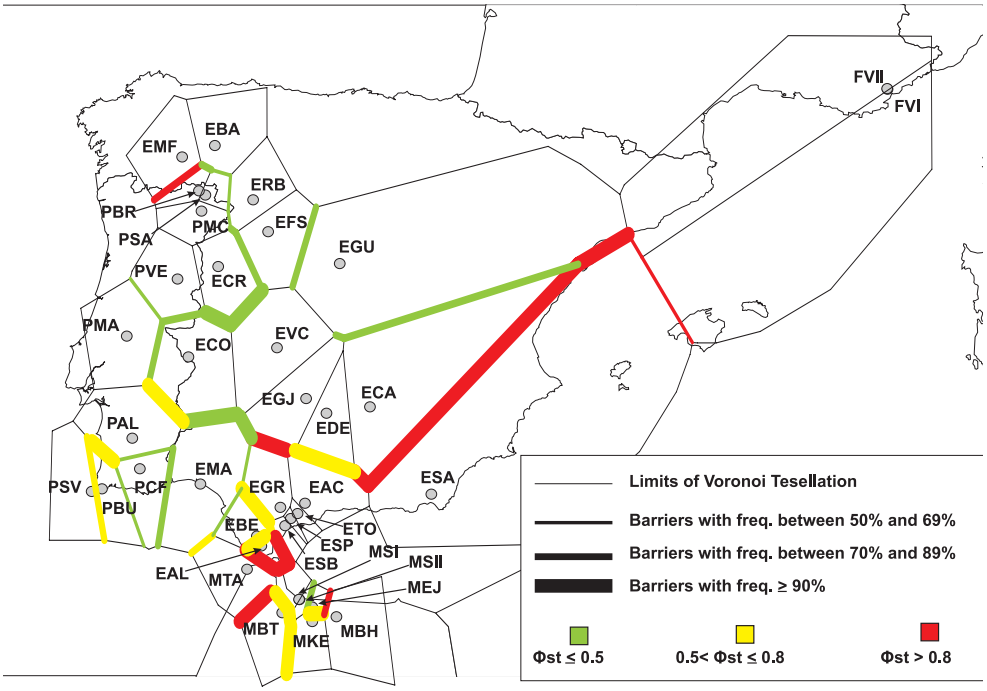

Figure 2.3: BARRIER results. Ten barriers were computed with Φ_{ST} pairwise distance matrices. The statistical support for each barrier (computed from the basis of 100 random matrices) is showed as line thickness. Only those barriers with a frequency higher than 50% were represented. The value of each barrier (Φ_{ST}) is indicated with a colour code (green, yellow, red). Populations are indicated by grey dots. The population code is indicated next to each dot (see Table 2.1). In some cases the population code is connected with an arrow to its dot.

new group comprising a single population from the southwest Iberian Peninsula (EMA) was identified (Φ_{CT} = 0.67). With value of K between 5 and 15, Φ_{CT} values did not increase significantly, and in most cases the newly defined groups comprised single populations.

Thus, SAMOVA analysis suggested the presence of three main geographic groups: one from North Morocco (hereafter Rif group), a group from the southeast Iberian Peninsula (hereafter Betic group) and a widespread group that occupies an area throughout the range of *C. ladanifer* (hereafter Western group); a fourth group is also present comprising a single population on the southwest coast of Spain (Huelva). The degree of population differentiation differed within each group (Table 2.2). Despite the short geographic distances among populations, the Rif group had the highest value of inter-population differentiation (Φ_{ST} = 0.42), whereas the widespread Western group had a lower differentiation value (Φ_{ST} = 0.32). No significant differentiation among populations within the Betic group was observed (Table 2.2). The analysis using pair-wise

Φ_{ST} distances in BARRIER software partially corroborated the SAMOVA analysis (Fig. 2.3). Although several barriers were inferred throughout the distribution area of *C. ladanifer*, the strongest genetic boundaries (with both high Φ_{ST} as well as high bootstrap support) were found in North Morocco (with barriers isolating single populations) and especially around the Betic area of the Iberian Peninsula. We stress that the BARRIER analyses separating the Iberian Peninsula and North Morocco were not inferred, although AMOVA analysis revealed that 25% of molecular variance was explained by genetic differences between populations north and south of the Strait of Gibraltar (Table 2).

Principal coordinate analysis (PCoA) extracted two axes that explained 100% of genetic variation (see Supplementary Material S 2.1). Plotting the populations along these axes revealed a grouping that matches the SAMOVA results: three groups (Betic, Rif and Western) were obtained, whereas the EMA population was plotted next to the populations from the Western cluster. This grouping has a certain taxonomic relevance. Population EBE, which belongs to *C. ladanifer* subsp. *africanus* is plotted in an intermediate position between Western and Rif group, whereas the two populations of subsp. *sulcatus* were placed together with populations of subsp. *ladanifer*.

2.4 Discussion

Chloroplast microsatellites have been widely used in phylogeographic studies (Magri *et al.* 2007, Fady *et al.* 2008, Pardo *et al.* 2008). Although some criticisms of cpSSRs arose due to problems with homoplasy, the level of homoplasy has been considered to be low enough to permit population genetic analysis (Provan *et al.* 1999a). Even when homoplasy has been identified, it was considered 'moderate' and its potential for invalidating results can be disregarded (Cuenca *et al.* 2003). Estoup *et al.* (2002), using simulations, concluded that the large amount of variability at microsatellite loci often largely compensates for homoplasious evolution, and cases in which size homoplasy may be a problem are related to high mutation rates. No estimate of mutation rates in *Cistus* is available but the lower mutation rate of chloroplast microsatellites compared to nuclear microsatellites (Provan *et al.* 1999b) suggests that size homoplasy may not be a major problem for studies at within-species level.

In this work *C. ladanifer* populations exhibited levels of genetic differentiation (G_{ST} = 0.60) that are similar to the mean and median values for maternally inherited markers in angiosperms (Petit *et al.* 2005a). Most of the molecular variance occurs between each of the four population groups, which exhibit high frequencies of haplotypes H2 (EMA), H7 (Betic group), H8 (Rif group) and H4 and H5 (Western group). From these haplotypes, it is possible that H8 is the more ancestral, since it has the largest number of connections with other haplotypes and is found in a more central position in the network (Posada and Crandall 2001). Furthermore, H8 gave rise to H7 and H4 haplotypes,

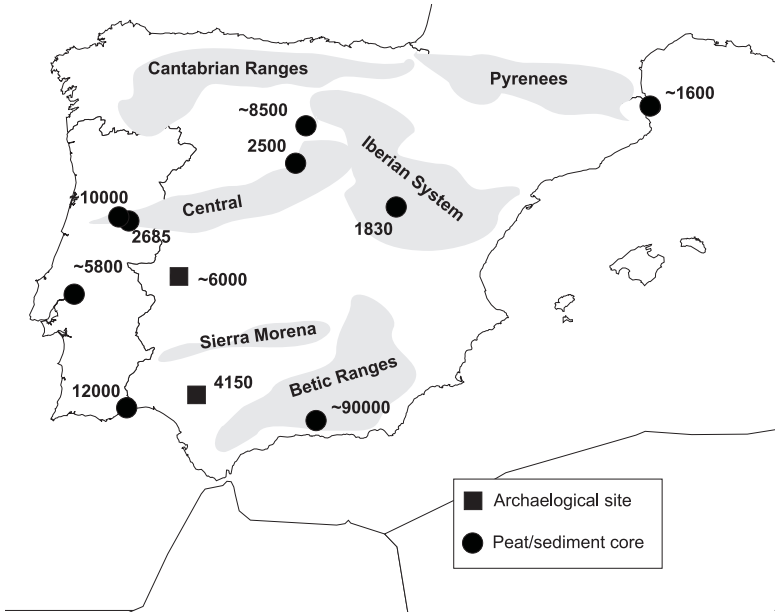

Figure 2.4: Sites and chronology of different deposits where samples of *C. ladanifer* pollen were recovered. Numbers next to each point indicate the chronology (years before present) of the first occurrence of C. ladanifer pollen in each site. The bibliographic sources of pollen data are reported in Supplementary material S 2.2.

which are the most frequent in the Betic and Western group of populations, respectively. These facts could point to the origin of the Betic and Western groups of *C. ladanifer* as a result of independent colonisation events from North Africa to the Iberian Peninsula, as proposed by Guzmán and Vargas (2009) based on chloroplast and nuclear sequence analysis.

The chronology of these colonisations is not clear, although the presence of *C. ladanifer* pollen in sites from the Betic area before the Last Glacial Maximum (around 90,000 years BP, Pons and Reille 1988, Fig. 2.4) and in southern Portugal during the Late Glacial (around 12000 years BP, Fletcher *et al.* 2007, Fig. 2.4) might indicate that it could have occurred during the Middle Pleistocene, a few million years after the opening of the Strait of Gibraltar at the end of the Messinian. Since opening (ca. 5.33 Ma, Hsü *et al.* 1977), the sea barrier of the Strait (14-km wide and about 400-m deep) has been maintained even during glacial maxima, although the lower sea level allowed emergence of different islands that reduced the width of the sea channels (Collina-Girard 2001): *C. ladanifer* should have been able to cross the Strait of Gibraltar using the emerging islands as stepping stones. The isolation effect caused by the Strait has left some traces on the genetic structure of *C. ladanifer* populations. First, three of the four groups of populations inferred by SAMOVA are present only on one side of

the Strait. Second, only two haplotypes (H3 and H8) are present both in Morocco and in the Iberian Peninsula. Finally, differentiation across the Strait accounts for 25% of the total molecular variance. However, the filtering effect of the Strait must have been lower than expected for a species like *C. ladanifer*, whose seeds tend to fall in a radius of 40 cm from the mother plant canopy (Bastida and Talavera 2002), given that the BARRIER analysis suggests that the Rif and Betic mountain ranges have acted as more effective barriers for seed flow than the Strait of Gibraltar.

The patterns of differentiation across the Strait of Gibraltar are diverse among different plant species. In a recent review, Rodríguez-Sánchez *et al.* (2008) found that establishment, rather than dispersal, may act as a key factor in genetic differentiation across the Strait. Thus, in spite of poor dispersal abilities, the high establishment potential of *C. ladanifer*, a species that can produce thousands of seeds per year that maintain viability for several years (3-year-old seeds have germination as high as 80%; personal observation, C. Quintela- Sabarís) could explain the relatively low effect of the Strait of Gibraltar on the genetic structure of its populations. In addition to the effect of the Strait, the high physiographic diversity of the northern part of Morocco (Rif Mountain ranges) and of the southern part of the Iberian Peninsula (river valleys and Betic ranges) created the geographic context for isolation during glacial periods, with the maintenance of pockets of Mediterranean taxa on south-facing slopes and in river gorges (Thompson 2005). Thus, in North Morocco (Rif region) and the south of Spain, four different clusters of populations are present. These clusters reflect the processes of post-glacial recolonisation of the Western Mediterranean area by *C. ladanifer* from its putative refugia. In the Rif region, we inferred a high degree of population differentiation and several barriers that delineate single populations (Fig. 2.3). This is congruent to an ancient presence of the species in this region and the effect of the Rif Mountains as barriers to seed flow. Regarding the Iberian Peninsula, our data suggest the occurrence of several independent glacial refugia instead of a single refugium area: first, the occurrence of three different clusters of populations (according to SAMOVA and PCoA), one of which is made up of population EMA with a unique haplotype at high frequency (0.6) on the southwest coast of Spain (this population is highly differentiated from Betic and Western clusters); second, the important genetic boundaries revealed by BARRIER analysis around the Betic region and especially in the Algeciras area (southernmost tip of the Iberian Peninsula) may indicate an area of contact between lineages expanding from different glacial refugia. In contrast with the high differences among populations in the southern Iberian Peninsula, only the Western group of populations is found in the northern part of the *C. ladanifer* natural range, suggesting that refugia in the southwest Iberian Peninsula were probably the only contributors to northward colonisation of this shrub. Populations in this area were

in a 'leading edge' position, since the lack of high mountain ranges and presence of siliceous soils in the southwest of the Iberian Peninsula favoured their expansion. The post-glacial expansion may have occurred rapidly, as illustrated by the early occurrence of *C. ladanifer* pollen in the centre of Portugal or in the Spanish Northern Meseta around 10,000–8000 BP (Van der Knaap and Van Leeuwen 1997, Franco-Mú gica *et al.* 2001, see Fig. 2.4). The presence of the related chlorotypes H4 and H5 in nearly all the populations of the Western cluster, together with the relatively low degree of population differentiation estimated among this group of populations, also support the rapid expansion model suggested by pollen data.

A stronger population genetic structure would be expected in a species with seeds dispersed mainly by barochory (Duminil *et al.* 2007), so the possible effect of endozoochory by red deer (*Cervus elaphus*; Malo and Suárez 1998) and even human activities should be taken into account as possible homogenising factors. *C. ladanifer* plays an important ecological role as coloniser in disturbed areas, so its expansion could be favoured by human-induced disturbances; thus the increase of *C. ladanifer* pollen recorded in several palaecological records is accompanied by other anthropogenic indicators, such as the increase of pollen from ruderal species (Van der Schriek *et al.* 2007) or even the decrease of *Olea* and evergreen oak pollen as a result of forest clearing by fire (Ló pez Sáez *et al.* 2007). Moreover, and related to changes in land use and microclimate variations, the presence of *C. ladanifer* pollen varied through different periods (e.g., reduction in *C. ladanifer* pollen in northeast Spain accompanied by an intense expansion of agriculture and forest cultivation from the 16th century AD; Ló pez-Sáez *et al.* 2009), a fact that could be linked to local extinctions/expansions of this plant that might have contributed to blurring of the original population genetic structure.

Moreover, *C. ladanifer* has been commercially exploited for centuries because of its fragrant resin (labdanum). The presence of *C. ladanifer* pollen in mummified remains from the 4th century AD found in Lyon (Girard and Maley 1999) as well as in cesspits from the 14th and 15th centuries in Flanders (Deforce 2006), is explained by its use as a cosmetic as well as in medicine. The intense use of *C. ladanifer* may have favoured human-mediated long-distance dispersal of this species in areas outside its natural distribution range. This may have occurred with *C. ladanifer* populations in the northeast Iberian Peninsula and southern France, where the hypothesis of artificial introduction of this species was formulated (Demoly and Montserrat 1993). Indeed, the populations analysed in southern France are fixed for the same chlorotype of populations as in the western part of the Iberian Peninsula. In contrast to expansion of the Western cluster, populations in the Betic area were encompassed by mountain ranges that acted as important barriers to the north, and may have expanded to the west, until they contacted other clusters of popula-

tions in the Algeciras area. Some authors (e.g., Petit *et al.* 2005b) proposed that colonisation of new territories might result in accelerated rates of molecular evolution and haplotype diversification, whereas the stable populations would retain ancestral characters. This could be an explanation for the lack of genetic structure, with fixation of haplotype H7 (which is directly related to the most ancestral haplotype H8) in almost all populations of this cluster. The Betic area has been identified as one of 10 hotspots of plant diversity in the Mediterranean Basin (Médail and Quézel 1997). In addition, at least two putative glacial refugia for plants have been identified in the southeast Iberian Peninsula (Médail and Diadema 2009). The latter work underlines the role of glacial refugia as climatically stable areas where we may find unique genetic diversity for plant species. Thus, for *C. ladanifer* (as for other species, such as the white oaks complex, Olalde *et al.* 2002 or *Pinus pinaster,* Bucci *et al.* 2007) the Betic area should be viewed as a 'relict' area where conservation of its populations (with a unique haplotype) should be a priority.

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Chapter 3

Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins

This chapter reproduces the published paper: Quintela-Sabarís C, Vendramin GG, Castro-Fernández D, Fraga MI (2010) Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins Plant and Soil 334:161-174

Previous page: *Cistus ladanifer* subsp. *africanus* growing in a degraded *Tetraclinis articulata* forest in the Bni Bouchra ultramafic area (Rif, N of Morocco). (Photo: C. Quintela-Sabarís)

CHAPTER 3

Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins

Keywords: Chloroplast microsatellites; *Cistus ladanifer*; genetic diversity; heavy metals; ultramafic areas

ABSTRACT

Cistus ladanifer L. (Cistaceae) is a Mediterranean shrub covering different kinds of soils in the Western Mediterranean area. This species has colonised several metalliferous areas (serpentine outcrops as well as human-polluted sites) throughout its distribution range, and is therefore an interesting species to study the possible effects on genetic diversity and differentiation produced by the colonisation of areas polluted with heavy metals. The genetic structure of 33 natural populations distributed across its entire natural distribution range (Morocco, Portugal and Spain) and growing on either metalliferous or non-metalliferous soils was investigated using chloroplast microsatellites. Population genetic parameters were estimated and genetic groups were identified using Bayesian inference. In addition, we compared the genetic diversity and differentiation among metallicolous and non-metallicolous populations within each Bayesian-defined group. The cpSSR data suggested that metallicolous populations of *Cistus ladanifer* have arisen through multiple independent evolutionary origins within two different chloroplast lineages. Evidence that the soil type provoked genetic bottlenecks in metallicolous populations or genetic differentiation among metallicolous and non-metallicolous populations was not observed. Historical factors are the main cause of the present genetic structure of *C. ladanifer*. The nature of tolerance to heavy metals as a species-wide trait in this shrub is discussed.

3.1 Introduction

Sites with high heavy metal contents in soils, either of natural origin (such as weathering of ultramafic bedrocks) or generated by anthropic activities (mining and industrial activities, atmospheric deposition, excessive use of agrochemicals or even highway traffic) (Padmavathiamma and Li 2007) are interesting areas for plant researchers due to their specific soil conditions and distinctive flora. Although some metals, such as Cu or Zn, are essential for their development, the occurrence of high contents of heavy metals has several toxic effects on plants: binding to proteins and alteration of their structure, displacement of essential elements resulting in deficiency effects or even promoting the formation of free radicals (Hall 2002). Metal toxicity, together with deficiency in nutrient contents, yield areas that are usually shallow with rocky soils and low moisture and with scarce plant cover (Brady *et al.* 2005). Thus, serpentine outcrops and mine deposits act as edaphic discontinuities in mainland regions, which have been defined as ecological or ‘edaphic islands’ (Lefèbvre and Vernet 1990).

When colonising these ‘islands’, plant populations have to cope with several environmental constraints (met-

als, dryness, isolation, etc.) that can leave their imprints on the genetic structure of plant populations. For instance, it has been proposed that plant populations in metalliferous areas can suffer a founder effect, which would significantly reduce their genetic diversity (Lefèbvre and Vernet 1990). Moreover, in some plant species, populations growing in metalliferous soils have shown significant genetic differentiation with respect to metal tolerance from neighbouring populations in 'normal' soils, even with the occurrence of substantial gene flow (Vékemans and Lefèbvre 1997, Linhart and Grant 1996 and references therein). Deng *et al.* (2007) using RAPD markers found significant genetic differentiation between mine populations and uncontaminated populations of the pseudometallophyte *Sedum alfredii*.

Plants growing in metalliferous soils can be either exclusive metallophytes (plants restricted to metalenriched habitats) or facultative metallophytes (also called pseudometallophytes), that is, species having both metallicolous (growing in metalliferous soils) and non-metallicolous populations (Wu 1990). Thus, pseudometallophytes are interesting species when studying the potential influence of environmental constraints on patterns of genetic diversity (Linhart and Grant 1996).

Several works have investigated the distribution of neutral genetic diversity within and between metallicolous populations (M) and non-metallicolous populations (NM) of different pseudometallophyte plant species (mainly herbaceous or undershrubs), using either isozymes (Wu *et al.* 1975, Ducousso *et al.* 1990, Westerbergh and Saura 1992, Bush and Barrett 1993, Vekemans and Lefèbvre 1997, Nordal *et al.* 1999, Nyberg Berglund and Westerbergh 2001) or DNA based markers (Mengoni *et al.* 2000, 2001, 2006, Pauwels *et al.* 2005).

In most cases, similar values of within-population diversity were estimated in M and NM populations, although in some works a diversity decrease was observed in M populations of *Deschampsia cespitosa* (Bush and Barrett 1993), *Lychnis alpina* (Nordal *et al.* 1999) and copper mine (but not serpentine) populations of *Silene paradoxa* (Mengoni *et al.* 2001).

Another subject that researchers have focused on, is the origin of the M populations. Analyses of diverse pseudometallophytes (Bush and Barrett 1993, Vekemans and Lefèbvre 1997, Mengoni *et al.* 2001, Pauwels *et al.* 2005) support the theory that geographically distant M populations (but even at short distances of hundreds of meters; Al-Hiyali *et al.* 1988) could have evolved independently from neighbouring NM populations; that is, M and NM populations do not constitute different phylogenetic lineages, and genetic distances among N and NM populations are a function of geographic distances between them.

These findings underline the importance of studying historical factors and population-genetic processes in order to dissect the effects of the demographic processes (such as patterns of migration or bottlenecks) from those related to selective processes (Staton *et al.* 2001).

Cistus ladanifer L. (gum rockrose) is a woody, semi-deciduous shrub growing in a wide range of latitudes, altitudes and climatic conditions in the Western Mediterranean region (South of France, Iberian Peninsula and North of Algeria and Morocco) (Demoly and Montserrat 1993). Its populations constitute early successional stages adapted to disturbances in Mediterranean ecosystems, in particular fires (Bastida and Talavera 2002). It is a pseudometallophyte that has established populations over different types of bedrock material (granites, schists, slates, etc.) and has also colonized different ultramafic areas in N Morocco (Bni Bouchra) (Ater *et al.* 2000), S Spain (Málaga) (Alados *et al.* 1999), NE Portugal (Trás-Os-Montes) (Díez Lázaro *et al.* 2006, Freitas *et al.* 2004a) and diverse mine tailings in Central to South-Western Iberian Peninsula (Murciego *et al.* 2007, Freitas *et al.* 2004b).

C. ladanifer is an entomophyllous, obligatory outcrossing species, bearing a gametophytic mechanism of incompatibility (Talavera *et al.* 1993). It is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). Three subspecies have been described based on leaf traits (Demoly and Montserrat 1993). Two subspecies, *Cistus ladanifer* subsp. *ladanifer* and subsp. *africanus*, are widespread and they have colonized M (ultramafic) areas, although only subsp. l*adanifer* is found also in mine tailings from the Iberian Peninsula. Finally *C. ladanifer* subsp. *sulcatus* (formerly *C. palhinhae*) is restricted to limestone derived soils on the coast of the southwestern tip of Portugal.

In this work, we have analysed 33 *Cistus ladanifer* populations sampled throughout the species distribution range using chloroplast (cp) DNA markers (microsatellites, SSRs). These markers are of special interest when studying colonisation patterns. Chloroplast DNA is generally maternally inherited in angiosperms, whose dispersion is therefore mediated by seeds only. In addition, the effective population size for haploid cpDNA is smaller than diploid nuclear genes, so the differentiation due to genetic drift can be stronger (Comes and Kadereit 1998) and phenomena like genetic bottlenecks can be more easily detected (Echt *et al.* 1998). For instance, cpSSR markers detected a reduction in genetic diversity within M populations of *Silene paradoxa* where RAPD markers failed (Mengoni *et al.* 2001).

These characteristics justify the wide use of cpDNA in order to infer the population history of plant populations (Petit *et al.* 2003, Magri *et al.* 2007). Once the phylogeography of the species has been inferred, a better understanding of the effect of metal pollution on the genetic structure of populations is possible, avoiding spurious correlations resulting from historical or demographic processes (Staton *et al.* 2001). This is especially interesting in *C. ladanifer*, since it colonizes M areas at different latitudes (from N Morocco to NE Portugal) in a region whose physiographic (spatial heterogeneity) and climatic diversity offers complex phylogeographic patterns (Gómez and

Lundt 2007).

In the present paper, the following main questions were addressed: Do M populations of *C. ladanifer* have the same origin? Did M populations of C*istus ladanifer* suffer a reduction in diversity? Do differences exist in the demographic effects of the colonisation of M areas along a latitudinal gradient?

3.2 Material and Methods

3.2.1 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire distribution natural range of this species were sampled. The subspecies growing in each site was identified on the basis of morphological traits. We included metallicolous (M) populations from different geographic areas: ultramafic outcrops of Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), and M populations growing on mine tailings from the centre of the Iberian Peninsula (Table 3.1). After the analyses of phyto-available trace metals, two populations (EAL and EDE), growing near highways, were included in the M group (see Fig. 3.1).

In each population, a longitudinal transect was established. Ten plants separated by at least 5 m were selected along the transect and their ripe fruits were collected. Seeds were sown and seedlings grown in a laboratory. One seedling per mother plant was selected for the subsequent analyses. Young plants were frozen in liquid nitrogen and conserved at −20°C until DNA extraction.

In addition, in each site one (or two) soil samples were collected from 5 to 15 cm in depth. Each soil sample was air-dried and sieved through a 2 mm-mesh.

3.2.2 Soil chemical analyses

Sieved soil subsamples were milled in an agatha mortar to achieve homogeneity. Total amounts of Cr, Cu, Mn, Ni, Pb and Zn in soils were quantified in solid subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF). Other subsamples were digested with HNO3 for the quantification of Co contents with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) element analysis.

In order to determine the concentration of heavy metals potentially available for plants, 10 g of dried soil was mixed with an extraction solution (Ammonium Acetate 0.5 M + EDTA 0.02 M + Acetic Acid 0.5 M, buffered at pH 4.65) (Lakanen and Erviö 1971) in a ratio of

Table 3.1: Within-population haplotypic diversity estimates. First column includes population name and code. Second column indicates *Cistus ladanifer* subspecies: *afr*: subsp. *africanus*; *lad*: subsp. *ladanifer*; *sul*: subsp. *sulcatus*. Geographic coordinates are given in decimal degrees. *M*: metallicollous population; (u): ultramafic area; (m): mine tailing; (h): highway affected area. *NM*: non-metallicollous population; *N.S.*: number of plants surveyed in each population; H_E: Nei's (1987) haplotypic diversity; $r_{(7)}$: haplotypic richness after rarefaction to the uniform sample size of 7 (El Mousadik and Petit, 1996); D^2_{SH}: average genetic distances among individuals (Vendramin *et al.* 1998); *Overall Mean*: mean for the whole set of populations ± standard deviation values.

Population (Code)	Subsp	Long	Lat	Soil Type	Substratum	N.S.	H_E	$r_{(7)}$	D^2_{SH}
Ketama (MKE)	*afr*	4.64º W	34.95º N	NM	Schists	10	0.38	1.40	0.22
Bni Hadifa (MBH)	*afr*	4.17º W	35.02º N	NM	Sandstones	10	0.2	0.70	0.1
Bab Tazaa (MBT)	*lad*	5.24º W	35.08º N	NM	Micaschists	10	0.2	0.70	0.1
El Jebha (MEJ)	*afr*	4.64º W	35.18º N	NM	Sandstone	10	0	0	0
East Bni Bouchra (MSII)	*afr*	4.89º W	35.29º N	M (u)	Serpentinised peridotite	12	0.7	2.16	0.58
West Bni Bouchra (MSI)	*afr*	4.90º W	35.30º N	M (u)	Serpentinised peridotite	10	0.53	1	0.27
Tanger (MTA)	*afr*	5.93º W	35.78º N	NM	Sandstone	10	0	0	0
Almodóvar (EAL)	*lad*	5.65º W	36.16º N	M (h)	Clays close to a road	10	0	0	0
Benalup (EBE)	*afr*	5.73º W	36.32º N	NM	Sandstone	10	0.64	2.33	0.76
Sierra Bermeja (ESB)	*lad*	5.18º W	36.48º N	M (u)	Serpentinised peridotite	10	0	0	0
Sierra Palmitera (ESP)	*lad*	5.07º W	36.60º N	M (u)	Serpentinised peridotite	9	0.42	1.56	0.5
Sierra de Tolox (ETO)	*lad*	4.93º W	36.68º N	M (u)	Serpentinised peridotite	10	0.2	0.70	0.1
Grazalema (EGR)	*lad*	5.27º W	36.78º N	NM	Decarbonated limestone	10	0	0	0
Sierra de Aguas (EAC)	*lad*	4.79º W	36.84º N	M (u)	Serpentinised peridotite	10	0	0	0
São Vicente (PSV)	*sul*	8.98º W	37.03º N	NM	Limestone	10	0.2	0.70	0.1
Burgau (PBU)	*sul*	8.78º W	37.07º N	NM	Limestone	9	0.39	0.97	0.19
Mazagón (EMA)	*lad*	6.84º W	37.15º N	NM	Sand deposits	10	0.62	1.87	1.78
Corte Figueira (PCF)	*lad*	8.03º W	37.39º N	NM	Schists	9	0.39	0.97	0.19
Aljustrel (PAL)	*lad*	8.18º W	37.88º N	M (m)	Pyrite mine tailing	9	0.64	1.78	0.53
Cardeña (ECA)	*lad*	4.36º W	38.28º N	NM	Granite	10	0.71	1.93	0.89
Despeñaperros (EDE)	*lad*	3.51º W	38.39º N	M (h)	Quartzites, close to the highway.	10	0.64	1.70	0.46
El Guijo (EGJ)	*lad*	4.77º W	38.52º N	NM	Slates	10	0.47	0.99	0.23
La Codosera (ECO)	*lad*	7.08º W	39.19º N	M (m)	Sb mine tailing	10	0.56	1	0.28
Valdecaballeros (EVC)	*lad*	5.34º W	39.33º N	NM	Sedimentary material (gravels, clays)	10	0.71	2.39	0.71
Vela (PVE)	*lad*	7.29º W	40.43º N	NM	Granite	9	0	0	0
Ciudad Rodrigo (ECR)	*lad*	6.49º W	40.63º N	NM	Quartzites	10	0.36	0.93	0.18
Guadarrama (EGU)	*lad*	4.10º W	40.68º N	NM	Granite	10	0.2	0.70	0.1
Fuente Saúco (EFS)	*lad*	5.51º W	41.19º N	NM	Sandstone and conglomerates	10	0.64	1.70	0.46
Macedo dos Cavaleiros (PMC)	*lad*	6.82º W	41.52º N	M (u)	Serpentinised peridotite	10	0	0	0
Ricobayo (ERB)	*lad*	5.81º W	41.70º N	NM	Quartzites and filites	10	0.53	1	0.27
Samil (PSA)	*lad*	6.75º W	41.78º N	M (u)	Serpentinised peridotite	7	0	0	0
Bragança (PBR)	*lad*	6.87º W	41.85º N	M (u)	Dunite	10	0.53	1	0.27
Monte Furado (EMF)	*lad*	7.20º W	42.39º N	NM	Schists	10	0	0	0
					Overall mean ± SD		0.31 ± 0.27	0.91 ± 0.77	0.27 ± 0.36

soil:extraction solution of 1:5. The suspension was shaken for 30 min, after which it was allowed to stand for at least half an hour and was then filtered through paper (Albet DP 145). The filtrate was stored cold to be analysed with an atomic absorption spectrophotometer (AAS). The following available trace metals were determined with AAS: Co, Cr, Cu Mn, Ni, Pb and Zn.

3.2.3 DNA extraction

DNA was extracted from 100 mg of frozen leaves using Dneasy® Plant Mini Kit (QIAGen), following the manufacturer's indications. In several cases an additional wash with 500 µl of absolute ethanol was needed in order to remove secondary compounds from the DNA extracts.

3.2.4 Microsatellite analysis

In an initial screening, universal cpSSR primers ccmp1 to ccmp10 (Weising and Gardner 1999) and Fagaceae cpSSR primers cmcs 1 to 14 (Sebastiani *et al.* 2004) were tested on a subset of 30 samples from 6 geographically distant populations. Only primers ccmp1, ccmp2, ccmp3, ccmp5, ccmp10 and cmcs1 yielded consistent amplifications, of which ccmp1, ccmp5, ccmp10 and cmcs1 were monomorphic. The two polymorphic cpSSRs were then used to amplify all samples of the 33 populations. Amplification reactions were performed in 12.5 µl total volume using 10 ng of template DNA, 1× reaction buffer (Promega, Madison, WI, USA) containing 1.5 mM of MgCl2, 0.2 µM of each primer, 0.2 mM of each dNTP, 1% of bovine serum albumin, and 0.5 U of GoTaq® DNA Polymerase (Promega). DNA was amplified with the following thermal profile: one denaturation cycle of 4 min at 95°C, followed by 25 cycles each consisting of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s with a final extension step at 72°C for 8 min. PCR products were loaded on a capillary automatic sequencer MegaBACE 1000 (GE Healthcare Biosciences). MegaBACE ET400 (GE Healthcare Biosciences) was used as size standard. Fragment lengths were determined using the MegaBACE FRAGMENT PROFILER software version 1.2 (GE Healthcare Biosciences).

3.2.5 Data analysis

A Principal Component Analysis (PCA) was performed in order to aggregate populations according to (1) total contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn in soils (referred here as CoT, CrT, CuT, MnT, NiT, PbT and ZnT) or (2) Ammonium Acetate/EDTA metal extractable contents (referred here as CoE, CrE, CuE, MnE, NiE, PbE and ZnE). Values below detection limits were recorded as 0.1 $\mu g.g^{-1}$ for statistical analysis. With the PCA, we reduced the dimensionality of the data, retaining, in our case, two first Principal Components (PCs) that contribute most to the variance of soils. A Varimax rotation was applied to the PCAs in order to simplify the interpretation of the extracted Principal Components.

Phylogenetic relationships among haplotypes were inferred with NETWORK 4.5 (Fluxus Technology Ltd. at www.fluxus-engineering.com) using the median

joining (MJ) method (Bandelt *et al.* 1999). We applied the three criteria (frequency, topology and geography) proposed by Pfenninger and Posada (2002) in order to remove loops or ambiguities in the haplotype network.

Different parameters of genetic diversity within populations were estimated: (1) the haplotypic diversity (H_E = $[n/(n-1)][1-\Sigma p_i^2]$, (where n is the number of individuals analysed in a population and p_i is the frequency of the i-th haplotype in a population; Nei 1987), (2) the haplotypic richness $r_{(n)}$, which is obtained after rarefaction to a uniform sample size of n (in our study, the value of n was fixed at 7, the lowest size of the analysed populations), as described in El Mousadik and Petit (1996), and (3) the D^2_{SH} measure, as defined by Vendramin *et al.* (1998), which takes into account the difference in the number of repeats among the different cpDNA haplotypes considered.

In addition, the distribution of the pairwise cpSSR repeat length differences among individual plants, totalled over all two cpSSR loci within an individual plant, was plotted to compare different patterns. Genetic differentiation among populations was estimated by the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) using Arlequin software (version 3.11; Excoffier *et al.* 2005). The significance of the values was computed by a permutation test from 10,000 permutated matrices. The AMOVA was based on distances between cpSSR haplotypes, calculated as the sum of the squared number of repeat differences between two haplotypes: $d_{xy} = \Sigma[a_{xi} - a_{yi}]^2$ (where a_{xi} and a_{yi} are the number of repeats for the ith locus in haplotypes x and y). This gives Φ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin 1995) for population differentiation (Michalakis and Excoffier 1996).

The Cavalli-Sforza and Edwards distances based on haplotype frequencies were used to construct a Neighbour Joining (NJ, Saitou and Nei 1987) dendrogram with the Populations software (O. Langella, UMR de Génétique Végétale, Ferme du Moulon, Gif/Yvette, France). To test for node robustness, bootstrapping was performed on individuals using 1,000 resamplings.

In order to infer population genetic structure, Bayesian analysis using a spatial clustering model implemented in BAPS software version 5.2 was performed (Corander *et al.* 2008). These authors have shown that the spatial model improves the statistical power to detect the underlying population structure when dealing with a low number of loci.

The spatial clustering of groups model was run using each population, with known coordinates, as the unit to be clustered. We initially fixed k (the number of clusters) from 2 to 25. We then selected the value of k that had the minimum log marginal likelihood and re-ran the analysis 100 times to obtain the optimal partition of populations. A neighbour-joining tree was then constructed (Saitou and Nei 1987) with the Kullback–Leibler divergence matrix provided as output with BAPS. This matrix can be used as a measure of relative genetic distance between the BAPS-identi-

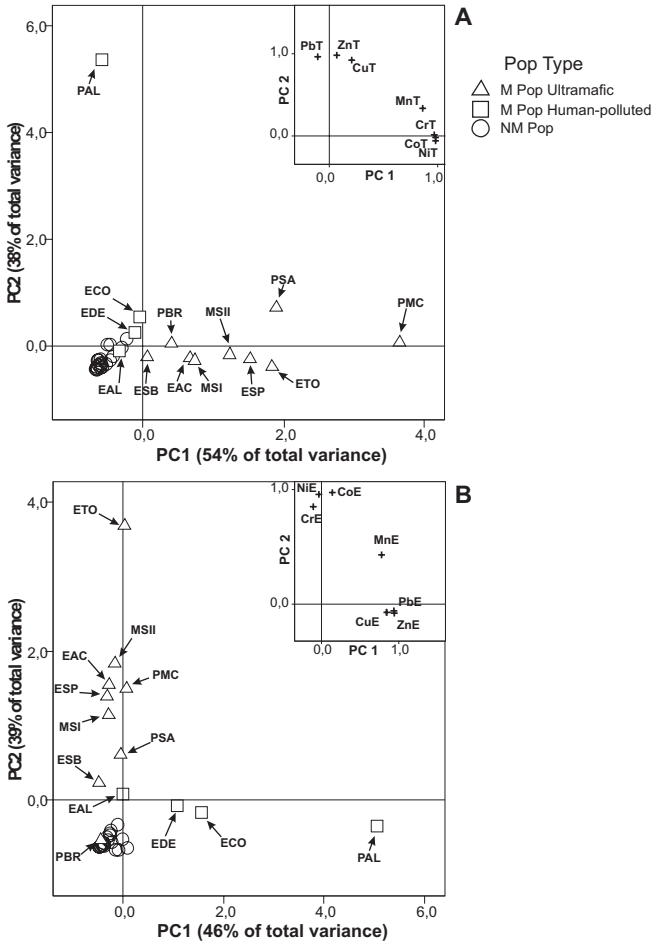

Figure 3.1: Principal Component Analysis (PCA) results. Populations are ordinated according to the total (Fig. 1a) or Ammonium Acetate/EDTA extractable (Fig. 1b) quantities of heavy metals in their soils. The percentage of variance explained by each axis is reported. Circles indicate non-metallicolous (NM) populations, whereas triangles and squares indicate metallicolous (M) populations from ultramafic areas and human-polluted soils, respectively. The codes of M populations are also indicated. In the upper right-hand corner of each graph the loading of each metal on each of the PCs is reported.

fied clusters (BAPS 5.2 manual distributed with the program).

Taking into account the grouping performed by BAPS, we (1) compared the levels of intra-population diversity of M and NM populations using an analysis of variance (ANOVA) and (2) tested the differentiation of M and NM populations with additional separate AMOVA analyses (Excoffier *et al.* 1992). With this two step-approach (BAPS and ANOVA/AMOVA) we tried to extract first the effect of phylogeography and then analyse the effect of colonisation of metallicolous areas within phylogeographic homogeneous groups of populations, thus avoiding confounding effects of phylogeography on the effect of metal pollution over population genetic structure.

Isolation-by-distance patterns between populations were tested considering all populations and then considering M and NM populations separately. A Mantel test with 10,000 random permutations was performed with the matrix of pairwise genetic differentiation between populations,

using $\Phi_{ST}/(1 - \Phi_{ST})$, and a matrix of the logarithmically (ln) transformed geographic distance. AMOVA and Mantel tests were computed with the Arlequin software version 3.11 (Excoffier *et al.* 2005).

3.3 Results

3.3.1 Soil characteristics

The results of the analyses of total and Ammonium Acetate/EDTA extractable metals in soils for each population are presented in the Supplementary Material S 3.1. On the basis of these data, we conducted two Principal Component Analyses (PCA).

The PCA performed on total metal contents (Fig. 3.1a) extracted two principal components (PCs) that explained 92% of total variance. The first Principal Component (PC1; 54% of total variance) was mainly composed of the total contents of Co, Cr, Ni, and Mn, suggesting that this axis is related to the ultramafic nature of soils. The second PC (PC2; 38% of total variance) was related to the degree of pollution due to human activities, since the metals with higher loadings were Cu, Pb and Zn.

The PCA based on extractable metals showed a similar pattern (Fig. 3.1b). Two PCs explaining 85% of total variance were extracted. In this case, the first PC (PC1; 46% of total variance) was related to human pollution (extractable Cu, Pb and Zn were the main contributors to this PC) whereas the second PC (PC2; 39% of total variance) reflected the ultramafic origin of soils, since the extractable contents of Co, Cr and Ni contributed significantly

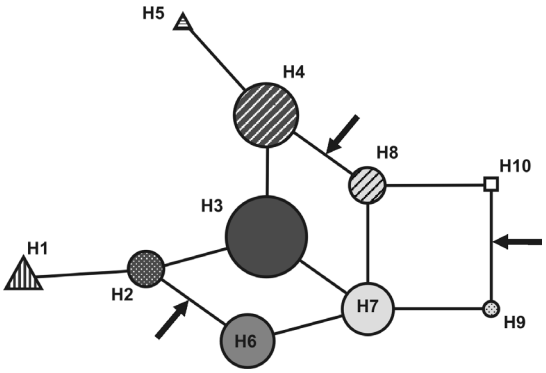

Figure 3.2: Median Joining (MJ) network of *Cistus ladanifer* cpSSR haplotypes. Haplotypes are identified with the same colours as in Fig.3.3. *Circles*: haplotypes present both in M and NM populations. *Triangles*: haplotypes exclusive to NM populations. *Square*: haplotype present only in M population. Symbols' sizes are proportional to the absolute haplotype frequency in the whole sample. Arrows indicate the connections between haplotypes that can be removed following the criteria of Pfenninger and Posada (2002).

in explaining the observed variance. The manganese content showed similar loadings on both axes.

According to the defined Principal Components, in both cases (total or extractable metal contents) NM populations were plotted in a dense swarm placed mainly in the negative values on both axes, whereas the M populations showed high scores along one of the PCs, depending on their nature (ultramafic area or human-polluted site) (Fig 3.1a and b).

On the other hand, three populations (EAL, EDE and PBR) showed differences between the two PCAs. Population PBR, whose bedrock material are dunites (a type of peridotite) is clearly separated from the NM populations based on total metal contents. Thus, it is plotted along

the axis of ultramafic populations in Fig 3.1a. In contrast, in Fig 3.1b (PCA based on extractable metal contents) this population is plotted with the NM populations. This population was treated as M because of the high total contents of metals Cr, Mn and Ni (944, 1,578 and 1,151 $\mu g.g^{-1}$, respectively) in the analysed soil samples.

The two other populations (EAL and EDE) were initially considered as NM (taking into account bedrock material and total metal contents, see Supplementary Material S 3.1 and Fig. 3.1a). Nevertheless, when performing the PCA on Ammonium Acetate/EDTA extractable metal contents, these two populations were separated from the group of NM populations (Fig 3.1b). These populations, affected by highway traffic, were consequently classified as M. EDE has an extractable Pb of more than 100 $\mu g.g^{-1}$, whereas EAL possesses in its soil moderate extractable contents of all the analysed metals.

3.3.2 Genetic diversity and structure

The two polymorphic microsatellites ccmp2 and ccmp3 yielded 3 and 5 size variants, respectively.
According to the nature of these microsatellites (both are mononucleotide repeats; Weising and Gardner 1999) the sizes of alleles varied by one base-pair.

The variants found in each microsatellite locus combined into 10 different haplotypes (see Supplementary Material S 3.2 for the definition of each haplotype). They are connected through a complex haplotype network, with 3 loops and no missing haplotypes (Fig. 3.2).

Only two haplotypes (H3 and H7) are present both in the Iberian Peninsula and North of Morocco. Haplotypes H2 to H4 are distributed across the Iberian Peninsula, whereas haplotypes H8 to H10 are found in North of Morocco and H6 is restricted to south-eastern Spain (Betic Area) (Fig. 3.3). Three haplotypes (H1, H5 and H10), found on the tips of the network, were exclusive to one population type (M or NM) (Fig. 3.2). Two of these haplotypes (H5 and H10) were singletons, whereas H1 is exclusive to NM population EMA from south-western Spain.

The geographic distribution and the frequency of haplotypes, together with a criterion of topology (Pfenninger and Posada 2002) were employed to remove one of the edges in each of the 3 loops inferred in the haplotype network and thus resolve the uncertainties in the network (Fig. 3.2).

Bayesian analysis yielded an ideal grouping with 8 clusters (Fig. 3.4), of which clusters 1, 3, 4, 6, and 7 included both M and NM populations. The NJ tree grouped these 8 clusters again in two diverging lineages of populations. The first lineage (hereafter referred to as 'South lineage') comprises populations in which H6 to H10 haplotypes are dominant (clusters 7, 8, 6 and 2), whereas the other lineage (hereafter referred to as 'North lineage') includes those populations with a high frequency of H1 to H5 haplotypes (clusters 4, 1, 3 and 5). These lineages have a taxonomic support, since the 'South lineage' comprises populations of *C. ladanifer* subsp *africanus*, together with populations

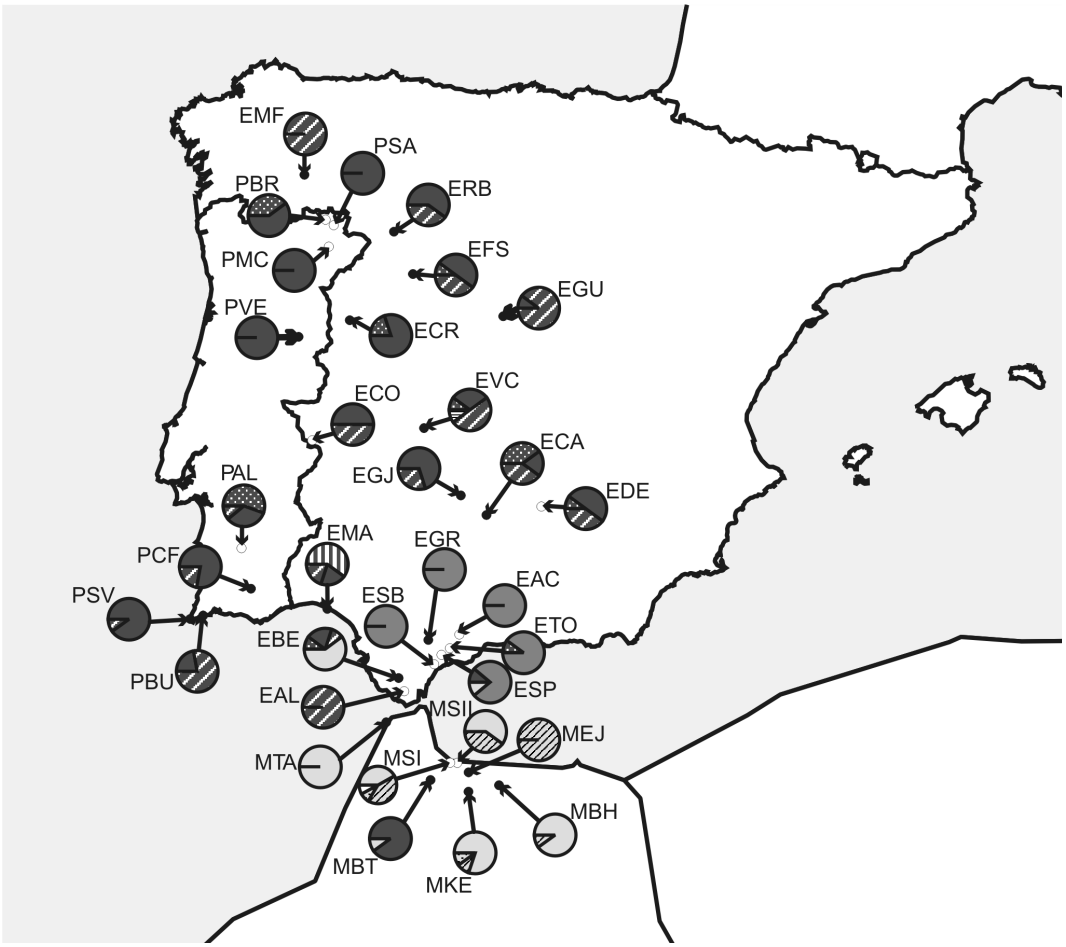

Figure 3.3: Geographic distribution of *Cistus ladanifer* cpSSR haplotypes. Colour patterns used are the same as in Fig 3.2. In order to obtain a proper view of haplotype distribution, radial graphs were displaced from their original position (arrows connect them with their original position). Next to each graph the population code is indicated (see Table 3.1). Metallicolous (M) populations are noted as white dots, non-metallicolous (NM) ones as black dots.

of *C. ladanifer* subsp. *ladanifer* growing in the Betic area, whereas the 'North lineage' is composed of populations of *C. ladanifer* subsp. *sulcatus* and *C. ladanifer* subsp *ladanifer* from the Iberian Peninsula, plus one population of subsp. *ladanifer* from the N of Morocco.

The AMOVA analysis performed on the whole sample of populations indicate that most of the molecular variation is found among populations (Φ_{ST}=0.69, Table 3.2a), that is a value similar to the mean value for angiosperms (Petit *et al.* 2005). Differentiation between edaphic types were not significant either when the whole set of populations (33 pops) was considered (Table 3.2b), nor when separate AMOVA analyses within each of the

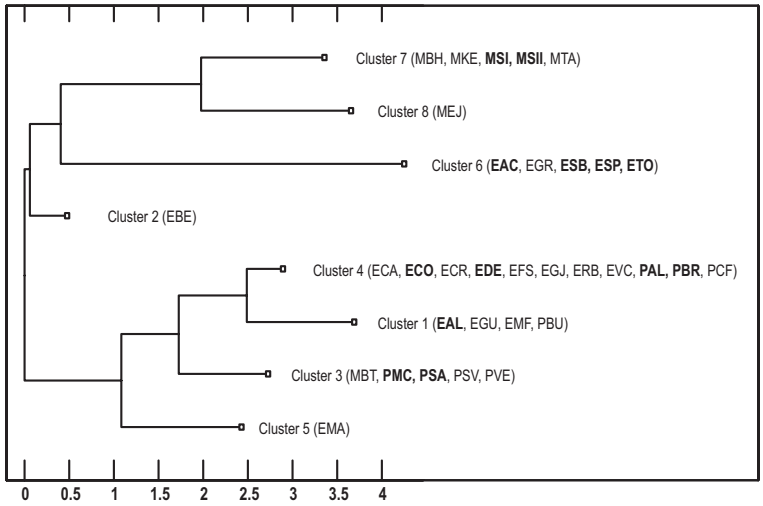

Figure 3.4: BAPS-based clustering and relationships among clusters based on the Kullback–Leibler divergence matrix. Clusters of populations were constructed with a spatial clustering model (Corander *et al.* 2008). Codes in brackets indicate populations included within each cluster. Population codes are the same as in Table 3.1. M populations are indicated in **bold type**. Horizontal scale bar indicates Kullback-Leibler distances among clusters.

lineages (North and South, Table 3.2c and d) were performed.

The NJ tree further confirmed that M and NM populations did not constitute distinct genetic groups (Fig. 3.5). M populations appear dispersed among NM populations, forming clusters that are partially congruent with BAPS results. In most cases, M populations are clustered with geographically close NM populations and were genetically distant to M populations from other geographic areas; on the other hand populations PMC, PSA and PVE (from the NE and C of Portugal) were clustered with population MBT from the N of Morocco.

No isolation-by-distance pattern was detected by Mantel's tests, irrespectively of whether we considered M populations ($P = 0.12$), NM populations ($P = 0.23$) or the whole set of populations ($P = 0.23$).

The number of haplotypes per population ranged from 1 to 4. The different estimators of within population diversity (H_E, $r_{(7)}$ and D^2_{SH}) displayed overall mean values of 0.31±0.27, 0.91±0.77 and 0.27±0.36, respectively (Table 3.1). The Analysis of Variance (ANOVA) showed no significant differences of diversity among M and NM populations considering either the whole set of populations or the lineages defined by BAPS (North, South) separately (Table 3.3). In addition, there were no qualitative differences among mismatch distributions from M and NM populations (see Supplementary Material S 3.3).

3.4 Discussion

Over the last 50 years pseudometallophytes have been studied as models of microevolution and of regional differentiation among plants. The main aim of these works has been, on the one hand, to test whether the colonisation of areas with heavy metals may result in genetically depauperated populations (as a result of

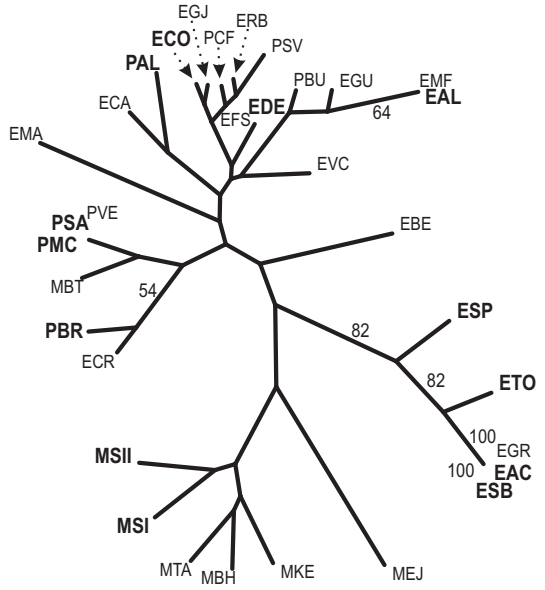

Figure 3.5: Neighbour-Joining consensus tree of populations. Cavalli-Sforza and Edwards distances between populations were used. Only bootstrap values higher than 50 are reported. M populations are indicated in **bold type**.

bottlenecks and genetic drift), and on the other, to determine whether the metalliferous populations of a particular species arose from a single micro-evolutionary event, or whether they resulted from processes of regional differentiation. Our study on the Mediterranean shrub *Cistus ladanifer*, which encompasses almost all of its natural distribution, provides evidence for the colonisation routes of metalliferous areas by this species.

Using Bayesian methods, we infer 2 population lineages with different haplotype compositions showing partial taxonomic significance ('South' lineage made up of the *C. ladanifer* subsp. *africanus* and subsp. *ladanifer* from the Betic area; 'North' lineage, made up of populations of *C. ladanifer* subsp. *ladanifer* and subsp. *sulcatus*).

The three *C. ladanifer* subspecies have been classified on the basis of leaf traits (leaf shape, nerve type and length of petiole, Demoly and Montserrat 1993). Our results showed an incongruence between molecular information and morphological traits: in fact populations of subspecies ladanifer from Betic area are placed within the cluster of subspecies africanus. This incongruence not only refers to chloroplast markers, but also to AFLP markers analysed on the same 33 populations included in this work (manuscript in preparation), thus suggesting that additional studies are needed to determine the taxonomic status for these subspecies.

In addition to the taxonomic considerations, several evidences indicate that the edaphic type does not influence the occurrence of the lineages: haplotypes specific to one single type of soil only appear on the tips of the haplotype network. Moreover, within the 'North' and 'South' lineages, as well as in the clusters defined by NJ dendrogram, both M and NM populations are found.

The two lineages detected in this study are congruent to those found by Guzmán and Vargas (2009) analysing the sequences of several chloroplast regions of *Cistus ladanifer*: these authors inferred two main chloroplast lineages, referred to as 'European' and 'African', and found that populations from the Betic area display an exclusive haplotype which belong to the 'African' lineage. Likewise, the two lineages we detected have the same

Table 3.2: Analysis of molecular variance (AMOVA) of *Cistus ladanifer*. We considered (a) all the populations (Φ_{ST}), (b) hierarchical analysis of variance among populations within edaphic types (Φ_{SC}) and between edaphic types (Φ_{CT}) and (c) (d) separate hierarchical analyses considering the clustering obtained by BAPS. *d.f.* = degrees of freedom, *SS* = sum of squared deviation, *Sig.* = probability of obtaining a more extreme component estimate by chance alone. *n.s.*, not-significant (*α value*=0.05)

Source of variation	d.f.	SS	Variance components	% Total Variance	Φ statistics	Sig.
(a) Whole data set						
Among populations	32	191.15	0.58	68.69	Φ_{ST} = 0.69	<0.0001
Within populations	291	77.14	0.26	31.31		
Total	323	268.29	0.85			
(b) M vs NM						
Among groups	1	10.16	0.03	3.23	Φ_{CT} = 0.03	n.s.
Among pops within groups	31	180.99	0.57	65.97	Φ_{SC} = 0.68	<0.0001
Within populations	291	77.14	0.26	30.80	Φ_{ST} = 0.69	<0.0001
Total	323	268.29	0.86			
(c) South cluster						
Between groups (M vs NM)	1	6.93	0.03	4.59	Φ_{CT} = 0.05	n.s.
Among pops within groups	10	50.32	0.48	71.62	Φ_{SC} = 0.75	<0.0001
Within populations	109	17.51	0.16	23.80		<0.0001
Total	120	74.76	0.67			
Among pops irrespective of groups					Φ_{ST} = 0.76	<0.0001
(d) North cluster						
Between groups (M vs NM)	1	0.36	-0.02	-4.63	Φ_{CT} = -0.04	n.s.
Among pops within groups	19	48.89	0.23	43.41	Φ_{SC} = 0.41	<0.0001
Within populations	182	59.63	0.33	61.22		
Total	202	108.88	0.53			
Among pops irrespective of groups					Φ_{ST} = 0.40	<0.0001

geographical distribution as reported by Guzmán and Vargas (2009); moreover haplotype H6, which is exclusive to populations from the Betic area, is connected to haplotype H7, common in the North of Morocco.

Thus, the 'North' and 'South' lineages seem to be more related to the existence of diverse glacial refugia for *Cistus ladanifer* located in the N of Morocco and in the Southeast (Betic area) and Southwest of the Iberian Peninsula, areas indicated as refugia for several other Mediterranean taxa (Médail and Diadema 2009).

Therefore, the extant M populations of *Cistus ladanifer* have arisen through distinct foundation events in different geographical regions and within different postglacial recolonisation lineages.

The independent origin of the M

populations of a given species seems to be common in pseudometallophytes (Mengoni *et al.* 2001, Nyberg Berglund and Westerbergh 2001, Vekemans and Lefèbvre 1997), even at a scale of hundreds of metres (Al-Hiyali *et al.* 1988). Interestingly, we found M populations within two chlorotype lineages while, for example, in *Arabidopsis halleri*, a model pseudometallophyte, all M populations, of independent origins, come from one chlorotype lineage only (north of the Alps) (Pauwels *et al.* 2005, 2008).

Most of the works on genetic structure of pseudometallophytes did not reveal evidence of genetic bottlenecks in M populations (for review, see Vekemans and Lefèbvre 1997, Mengoni *et al.* 2000). These results were mainly based on nuclear markers (isozymes or RAPDs), so the possible founder effect at nuclear loci may be eroded by subsequent pollen flow from neighbouring NM populations which could increase genetic variation in M populations. In contrast, chloroplast markers are (generally) maternally inherited, so they will only reflect the effect of seed flow. To our knowledge, only two other studies have analysed pseudometallophytes using chloroplast markers, obtaining contrasting results: Mengoni *et al.* (2001) detected a founder effect in *Silene paradoxa* populations growing on copper mine deposits, whereas Pauwels *et al.* (2005) did not find any difference in genetic diversity between M and NM populations of *Arabidopsis halleri*.

To explain these different patterns of diversity between *S. paradoxa* and *A. halleri*, Pauwels *et al.* (2005) proposed that the colonisation of metalpolluted environments is associated with a genetic bottleneck in species with populational tolerance (that is, species in which metal tolerance is found only in those populations growing on metalliferous soils), whereas in species with constitutive (or "specieswide") tolerance (such as *A. halleri*) the effect of a bottleneck may not be detected.

In the case of *Cistus ladanifer*, once we had extracted the phylogeograph-

Table 3.3: ANOVA results. The table presents the mean values for M (Metallicolous) and NM (Non-Metallicolous) of within-population diversity indexes for each cluster of populations (defined after Bayesian analysis), and for the whole set of populations. H_E: Nei's (1987) haplotypic diversity. $r_{(7)}$: allelic richness (El Mousadik and Petit, 1996) with a fixed rarefaction size of 7. D^2_{SH}: average genetic distances among individuals (Vendramin *et al.* 1998). *Sig.*: significance value. *n.s.*: not-significant (*α value*= 0.05).

		H_E			$r_{(7)}$			D^2_{SH}		
		M	**NM**	**Sig.**	**M**	**NM**	**Sig.**	**M**	**NM**	**Sig.**
Cluster defined by BAPS	South	0.308	0.203	n.s.	0.903	0.738	n.s.	0.242	0.180	n.s.
	North	0.339	0.387	n.s.	0.783	1.061	n.s.	0.220	0.371	n.s.
Whole data		0.325	0.332	n.s.	0.838	0.964	n.s.	0.230	0.314	n.s.

ic effect through the Bayesian analysis, we did not detected any significant differentiation nor significant differences in the genetic diversity between edaphic types, even in the M populations of more recent origin, i.e. populations growing on mine tailings from the Iberian Peninsula.

These inferences may suggest that M populations were founded recently by a high number of individuals, or that the foundation events are antique but in the presence of a significant seed flow (cpDNA is maternally inherited in C*istus ladanifer*; Guzmán and Vargas 2009) from neighbouring NM populations that masked the effect of genetic bottlenecks and limited genetic differentiation between edaphic types.

Both hypotheses assume that the metalliferous areas do not exert selective pressures on *Cistus ladanifer*; however, it should be stressed that variation of neutral markers, like cpSSRs, cannot be related to variation of adaptive traits such as tolerance to heavy metals (Le Corre and Kremer 2003), as it occurred in *Thlaspi caerulescens* (Jiménez-Ambriz *et al.* 2007). Thus, genetic differentiation between M and NM populations at genes related to metal-tolerance can be significantly underestimated.

Nevertheless, as discussed by Vekemans and Lefèbvre (1997), after a bottleneck the number of alleles at neutral loci increases as a result of new mutations, when population size increases. This phenomenon can be observed in the pseudometallophyte *Silene paradoxa* (Mengoni *et al.* 2001): 8 populations (5 serpentine outcrops, 2 copper mines, 1 nonmetallicolous) separated up to 205 km showed 13 cpSSR haplotypes exclusive to one population (of 27 haplotypes detected) and no haplotypes were shared by all populations. In contrast, in the case of *C. ladanifer* only a singleton was exclusive of M populations. This fact suggests that the inferred levels of diversity cannot be caused by mutations after a bottleneck following the colonisation of metalliferous areas.

To conclude, following Pauwels *et al.* (2005) and considering the multiple origins of M populations and the lack of bottlenecks, we propose that the tolerance to heavy metals could be a characteristic of *Cistus ladanifer*, even though this plant is more commonly found in non-metalliferous areas, as already observed in *Thlaspi montanum* (Boyd and Martens 1998) or in *Andropogon virginicus* (Gibson and Risser 1982). Indeed, our hypothesis is supported by the results obtained by Kidd *et al.* (2004), who observed that nonmetallicolous populations from North of Portugal showed high tolerance to Cu and Zn in hydroponic cultivation.

Taking into account our findings on *Cistus ladanifer* and those obtained by other authors, we believe that this plant could be very useful in the recovering of degraded soils in the Mediterranean region, also considering that it has other interesting traits (Frérot *et al.* 2006). First, *C. ladanifer* is adapted to water stress (Nuñez-Olivera *et al.* 1996) so it can cope with a long, dry summer season. Second, it has a high growth rate and productivity (Patón *et al.* 1998) when conditions

permit, developing dense root and shoot systems that can limit soil erosion. And third, it is a native species of Western Mediterranean flora; therefore, its use in phytoremediation would not imply any potential threats to ecosystems deriving from the use of alien species (Méndez and Maier 2008; and references therein), an important issue in the biodiversity-rich Mediterranean area.

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Chapter 4

Heavy metal accumulation in leaves of divergent chloroplast lineages of the pseudometallophyte *Cistus ladanifer* L. Implications for phytostabilization

Previous page: General view of an old pyrite mine tailing in Aljustrel (Alentejo, S Portugal). In this tailing we quantified (total contents): 752 $mg \cdot kg^{-1}$ As; 374 $mg \cdot kg^{-1}$ Cu; 1098 $mg \cdot kg^{-1}$ Mn; 2347 $mg \cdot kg^{-1}$ Pb and 633 $mg \cdot kg^{-1}$ Zn. *Cistus ladanifer* subsp. *ladanifer* has naturally colonised this tailing and now it is the dominant species. (Photo: C. Quintela-Sabarís)

CHAPTER 4

Heavy metal accumulation in leaves of divergent chloroplast lineages of the pseudometallophyte *Cistus ladanifer* L. Implications for phytostabilization

Keywords: Accumulation, *Cistus ladanifer*, heavy metals, pseudometallophyte

ABSTRACT

Cistus ladanifer is a shrub which grows in different kinds of soils (including serpentine outcrops and human-polluted sites) in the Western Mediterranean area. Chloroplast DNA analyses have inferred that metallicolous populations of this plant have arisen through distinct foundation events within two different postglacial recolonisation lineages.

We have quantified the levels of Co, Cr, Cu, Mn, Ni, Pb and Zn in soils and in leaves of *C. ladanifer* plants from 33 populations, covering almost its entire natural range. In addition, we have computed the ratio between metal contents in leaves and in soils, as a measure of accumulation abilities. Through a nested analysis of variance (nANOVA) we tried to evaluate whether the population type (metallicolous vs. non-metallicolous) or the chloroplast lineage ('North' vs. 'South') differ in their metal contents and accumulation patterns.

Our results show that, on a broad scale, metallicolous populations have higher Ni contents than non-metallicolous ones ($p<0.001$) and lineage 'North' higher Mn contents than lineage 'South' ($p<0.001$). In addition, Cistus ladanifer clearly rejects the accumulation of Co, Cr and Pb in its leaves, whereas for the other metals there were different accumulation patterns between lineages and population types.

The implications of our results in the use of *Cistus ladanifer* for phytostabilization procedures are discussed.

4.1 Introduction

Areas with high contents of heavy-metals in soils are the result of natural processes (weathering of ultramafic rocks) or human activities (mining and industrial activities, atmospheric deposition, excessive use of agrochemicals or even traffic emissions).

Several technologies have been employed in order to remediate human polluted soils, the most of them based on expensive mechanical soil treatments that sometimes include soil removal and replacement. In recent years, phytoremediation, that is, the use of different plant species for soil remediation, has been proposed as an environmentally friendly technology that in addition has a lower economic cost than traditional approaches (Padmavathiamma and Li 2007).

Plant growth is inhibited in the most severely contaminated sites, so human and animal exposure to heavy metals can be increased by the migration of contaminated soil (erosion, dispersal by wind) or leaching into groundwater (Ruttens *et al.* 2006).

In sites with high and multi-elemental contamination, phytostabilization (the use of native or introduced plants to transform soil metals to less toxic forms, but not remove the metal from the soil, Chaney

Table 4.1: Description of *Cistus ladanifer* populations included in this work.

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with dispersed *Pinus pinea* and *P. halepensis* trees	4.79º W	36.84º N
Almodóvar (EAL)	North	M (h)	Clays close to a road	Grassland with dispersed *C. ladanifer* plants	5.65º W	36.16º N
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N
Despeñaperros (EDE)	North	M (h)	Quartzites, close to the highway.	Dense *Cistus ladanifer* shrubland with *Quercus ilex* and *Juniperus oxycedrus*	3.51º W	38.39º N
Fuente Saúco (EFS)	North	NM	Sandstone and conglomerates	Shrubland with *Quercus ilex*	5.51º W	41.19º N
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N
Grazalema (EGR)	South	NM	Decarbonated limestone	Shrubland with *C. ladanifer* and *C. monspelliensis*	5.27º W	36.78º N
Guadarrama (EGU)	North	NM	Granite	Dense shrubland with isolated *Quercus ilex*	4.10º W	40.68º N
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N
Valdecaballeros (EVC)	North	NM	Sedimentary material (gravels, clays)	Dense shrubland with *Pinus pinaster*	5.34º W	39.33º N
Bni Hadifa (MBH)	South	NM	Sandstones	Open *Pinus halepensis* forest	4.17º W	35.02º N
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N
El Jebha (MEJ)	South	NM	Sandstone	Open *Pinus halepensis* forest	4.64º W	35.18º N
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks	4.64º W	34.95º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

Table 4.1: (continued)

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89º W	35.29º N
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N
Tanger (MTA)	South	NM	Sandstone	Open *Pinus pinaster* forest	5.93º W	35.78º N
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18º W	37.88º N
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87º W	41.85º N
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78º W	37.07º N
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03º W	37.39º N
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82º W	41.52º N
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75º W	41.78º N
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98º W	37.03º N
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29º W	40.43º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

et al. 1997) is the most suitable method of remediation (Kidd *et al.* 2009).

As the plants cover the soil surface, they prevent erosion, reduce water percolation and increase biodiversity (Brown *et al.* 2005). In addition, the biological activities and the production of organic matter by the vegetation may contribute to metal immobilization (Vangrosveld *et al.* 2009).

Identification and characterization of plant species capable of growing and surviving in polluted areas could be very helpful in developing phytostabilization technologies. Ideal plant species should have a rapid growth rate and dense root and shoot systems. Moreover, this species should not accumulate metals into above-ground tissues to prevent wildlife exposure and surface contamination.

In addition, the plants used in phytostabilization work in the Mediterranean region should be adapted to water stress in order to cope with a long dry summer season (Frérot *et al.* 2006). Given the fact that the introduction of alien (and potentially) invasive species could produce detrimental effects on the surrounding ecosystems (Méndez and Maier, 2008; and references therein), the use of native plants for local flora in phytostabilization procedures should be a priority.

Cistus ladanifer L. (Cistaceae) is a woody, semideciduous shrub found growing in a wide range of latitudes, altitudes, climatic conditions and soil types in the Western Mediterranean region. Its populations constitute early successional stages adapted to disturbances operating in Mediterranean ecosystems, especially fire (Bastida and Talavera 2002). Moreover, this species retains potentially active leaves through the summer drought (Núñez-Olivera *et al.* 1996). It has been found that natural populations of this plant can produce up to 4,600 kg of dry matter $ha^{-1}.year^{-1}$ of litterfall that improves soil quality (Simões *et al.* 2009). In addition, it develops dense root and shoot systems that can limit the erosion of soil (Martín Bolaños and Guinea López 1949).

This species is a pseudometallophyte. Thus, it is present in non-metalliferous soils and also in metalliferous areas (ultramafic outcrops and also mine tailings) from the North of Morocco to NW of the Iberian Peninsula (Alvarenga *et al.* 2004, Ater *et al.* 2000, Batista 2003, Díez-Lázaro *et al.* 2004, Freitas *et al.* 2004, Pratas *et al.* 2005). In these sites, *C. ladanifer* behaves as an indicator, or even an accumulator, of different heavy metals. In previous work using chloroplast DNA markers, (Quintela-Sabarís *et al.* 2010) we inferred that metallicolous populations of this plant have arisen through distinct foundation events within two different postglacial recolonisation lineages.

Given the fact that variations in tolerance and accumulation capacity are genetically controlled, metallicolous populations with independent origins might show different patterns of response to heavy metals, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Cerastium alpinum* (Nyberg Berglund *et al.* 2003) or *Thlaspi caerulescens* (Assunção *et al.* 2003).

The aim of this study was to analyse the Co, Cr, Cu, Mn, Ni, Pb and Zn leaf contents of field-collected plants from numerous metallicolous and non-metallicolous populations of *C. ladanifer* from two diverging chloroplast lineages in order to explore the extent and variability of metal accumulation in natural populations of the species and thus determine if any populations are more useful for phytostabilization procedures. To compare the metal accumulation abilities of plants originating from metalliferous and non-metalliferous soils, ratios between metal content in leaves and in soils were calculated (Bert *et al.* 2002). More precisely, the following questions were addressed: (i) Do the metallicolous (M) and non-metallicolous (NM) populations differ in metal accumulation? and (ii) Do the M and NM populations from different chloroplast lineages show differences in metal accumulation patterns?

Table 4.2: Heavy metal contents in leaves of *Cistus ladanifer*. The mean ± SE is presented for each population. Last three rows indicate the overall mean, maximum and minimum contents measured in the leaves. Values are expressed in $\mu g.g^{-1}$ of dry weight. Population codes in **bold type** indicate those which we considered as metallicolous (M). Population codes are the same than Table 4.1.

Pop	Co	Cr	Cu	Mn	Ni	Pb	Zn
EAC	1.6 ± 0.9	24.1 ± 5.6	10.7 ± 2.4	60.5 ± 7.7	36.9 ± 5.2	1.2 ± 0.8	75.2 ± 9.8
EAL	5.5 ± 4.5	28.8 ± 9.2	13.4 ± 2.9	734.1 ± 544.2	20.0 ± 5.4	0.4 ± 0.5	123.5 ± 27.7
EBE	5.8 ± 4.5	23.1 ± 3.4	11.0 ± 2.3	316.3 ± 107.6	11.8 ± 1.9	0.5 ± 0.4	89.8 ± 31.2
ECA	2.3 ± 0.9	25.5 ± 12.3	8.9 ± 2.1	539.5 ± 323.9	10.2 ± 2.9	0.9 ± 0.4	105.5 ± 107.9
ECO	2.8 ± 2.1	25.5 ± 12.2	12.4 ± 3.7	1688.5 ± 1324.6	19.3 ± 5.9	4.2 ± 2.3	88.2 ± 15.0
ECR	2.4 ± 2.0	23.0 ± 4.2	13.3 ± 3.6	1078.1 ± 799.6	17.1 ± 5.5	0.1 ± 0.1	86.1 ± 9.1
EDE	5.7 ± 2.7	24.9 ± 8.1	14.9 ± 3.4	865.1 ± 253.2	15.9 ± 2.9	0.7 ± 0.5	97.1 ± 18.0
EFS	3.7 ± 3.0	21.1 ± 5.2	15.5 ± 4.4	1135.4 ± 374.9	32.3 ± 2.8	0.2 ± 0.3	122.1 ± 36.5
EGJ	3.3 ± 1.6	21.7 ± 2.7	11.1 ± 1.7	914.6 ± 462.6	18.6 ± 4.6	1.2 ± 0.4	95.4 ± 9.7
EGR	0.8 ± 0.3	22.7 ± 3.3	14.9 ± 4.2	310.4 ± 100.5	17.8 ± 4.2	0.1 ± 0.1	100.3 ± 15.7
EGU	0.7 ± 0.4	20.8 ± 3.1	10.8 ± 2.4	523.8 ± 257.4	10.2 ± 2.8	0.5 ± 0.8	102.3 ± 20.2
EMA	2.6 ± 1.6	28.4 ± 9.2	37.4 ± 6.9	264.3 ± 88.5	14.8 ± 1.7	0.7 ± 0.6	128.7 ± 33.9
EMF	2.5 ± 0.6	25.8 ± 4.1	12.8 ± 4.4	344.5 ± 235.8	18.0 ± 4.8	0.3 ± 0.3	93.9 ± 26.4
ERB	4.7 ± 1.0	29.4 ± 8.5	8.3 ± 0.8	483.0 ± 149.5	18.0 ± 2.7	0.2 ± 0.4	143.4 ± 86.0
ESB	7.8 ± 8.9	29.3 ± 11.0	8.5 ± 1.7	275.6 ± 331.9	50.0 ± 16.6	0.3 ± 0.3	79.0 ± 30.7
ESP	1.5 ± 0.8	31.2 ± 7.1	8.3 ± 1.3	117.5 ± 39.9	58.8 ± 17.9	0.1 ± 0.2	76.6 ± 14.3
ETO	2.0 ± 0.7	27.3 ± 7.3	6.6 ± 0.9	98.7 ± 14.2	75.7 ± 7.5	0.3 ± 0.3	63.3 ± 6.6
EVC	2.3 ± 1.3	30.2 ± 6.5	8.6 ± 2.3	805.2 ± 343.9	14.4 ± 3.1	0.3 ± 0.3	108.6 ± 32.7
MBH	3.2 ± 1.7	23.8 ± 5.3	13.5 ± 3.8	336.9 ± 80.2	12.6 ± 2.0	0.3 ± 0.4	86.3 ± 19.1
MBT	3.8 ± 1.6	25.4 ± 4.0	10.5 ± 3.2	1179.0 ± 573.4	18.7 ± 4.4	0.4 ± 0.4	93.5 ± 28.1
MEJ	1.5 ± 1.1	30.8 ± 15.4	8.5 ± 0.5	264.0 ± 148.1	12.5 ± 4.6	0.1 ± 0.2	92.7 ± 13.8
MKE	4.7 ± 2.5	25.5 ± 6.4	12.6 ± 2.8	554.9 ± 120.1	15.7 ± 2.5	0.2 ± 0.2	85.6 ± 20.8
MSI	1.0 ± 0.7	26.2 ± 7.6	8.2 ± 1.1	51.6 ± 27.4	47.1 ± 6.8	0.5 ± 0.5	66.1 ± 9.1
MSII	1.2 ± 0.2	25.3 ± 3.8	7.3 ± 1.1	59.3 ± 25.3	53.7 ± 12.8	0.5 ± 0.4	73.8 ± 8.3
MTA	1.5 ± 0.7	25.2 ± 4.8	12.5 ± 4.1	78.8 ± 37.5	10.9 ± 1.9	0.2 ± 0.2	83.9 ± 27.7
PAL	6.7 ± 2.4	27.2 ± 8.0	26.6 ± 3.2	980.7 ± 385.0	25.7 ± 6.4	1.7 ± 0.9	288.0 ± 94.5
PBR	2.1 ± 1.5	25.7 ± 4.0	9.3 ± 2.7	232.6 ± 43.5	74.5 ± 13.8	0.3 ± 0.4	85.1 ± 16.2v
PBU	0.6 ± 0.5	23.3 ± 4.9	9.8 ± 2.5	174.5 ± 169.7	8.3 ± 1.4	0.4 ± 0.4	56.8 ± 14.6
PCF	5.4 ± 2.5	22.4 ± 6.5	12.2 ± 2.9	1485.8 ± 414.8	18.7 ± 2.9	0.2 ± 0.2	57.5 ± 8.0
PMC	2.6 ± 2.0	24.7 ± 7.1	12.5 ± 1.4	308.1 ± 338.6	99.6 ± 41.6	0.2 ± 0.3	87.0 ± 9.5
PSA	1.5 ± 0.3	50.0 ± 9.1	8.1 ± 0.8	186.2 ± 62.2	93.1 ± 28.3	0.3 ± 0.3	77.5 ± 18.4
PSV	0.3 ± 0.2	24.5 ± 5.2	10.8 ± 2.2	52.4 ± 15.2	9.7 ± 2.4	0.3 ± 0.3	78.2 ± 34.1
PVE	1.9 ± 1.1	20.0 ± 4.7	9.6 ± 2.3	809.6 ± 518.3	10.2 ± 2.3	0.1 ± 0.1	71.9 ± 20.8
Overall Mean	2.9	26.2	12.1	517.1	29.4	0.5	95.7
Max.	25.1	61.12	46.13	3952.3	141.3	8.1	423.7
Min.	0.03	11.32	4.43	20.71	6.1	0	35.2

4.2 Material and methods

4.2.1 Study species

Cistus ladanifer is an entomophyllous, obligatory outcrossing species, bearing a gametophytic mechanism of incompatibility (Talavera *et al.* 1993). It is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). Three subspecies have been described based on leaf traits (Demoly and Montserrat 1993). Two subspecies, *Cistus ladanifer* subsp. *ladanifer* and subsp. *africanus*, are widespread and they have colonized metalliferous (ultramafic) areas, although only subsp. *ladanifer* is found also in mine tailings from the Iberian Peninsula. Finally *C. ladanifer* subsp. *sulcatus* (formerly *C. palhinhae*) is restricted to limestone-derived soils on the coast of the south-western tip of Portugal.

4.2.2 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire natural range of this species (and its three subspecies) were sampled. In previous work (Quintela-Sabarís *et al.* 2010), on the basis of soil metal contents (either Total or Ammonium Acetate/Acetic Acid/EDTA extractable contents) we have classified these populations as metallicolous (M) and non-metallicolous (NM). M populations included those growing on ultramafic outcrops in Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), or on human-polluted soils, such as mine tailings, or areas affected by highway traffic.

In addition, using chloroplast microsatellites (cpSSRs) we have inferred that M and NM populations belong to two independent lineages ('North' and 'South') that were isolated during last Glacial period (Quintela-Sabarís *et al.* 2010).

In each population, a longitudinal transect was established, along which six plants, separated by at least 5m, were selected. We collected 5 branches with leaves from each of the selected plants. At the laboratory, leaves were separated from stems, and they were cleaned with fresh water, rinsed twice with distilled-deionised water and dried at 60ºC for two days. In addition, in each site soil samples were collected from 5-15 cm in depth. Each soil sample was air-dried and sieved through a 2mm-mesh.

4.2.3 Chemical analyses

Dried leaves and soil samples were ground to achieve homogeneity. Soil Total and extractable metal contents were quantified previously (Quintela-Sabarís *et al.* 2010).

The amounts of Cr, Cu, Mn, Ni, Pb and Zn in leaves were quantified in solid subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF). Other subsamples were digested with HNO_3 for the quantification of Co contents with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) element analysis.

Any value lower than the detection limit was recorded as 0.1 $\mu g \bullet g^{-1}$ for statistical analyses (Bert *et al.* 2002).

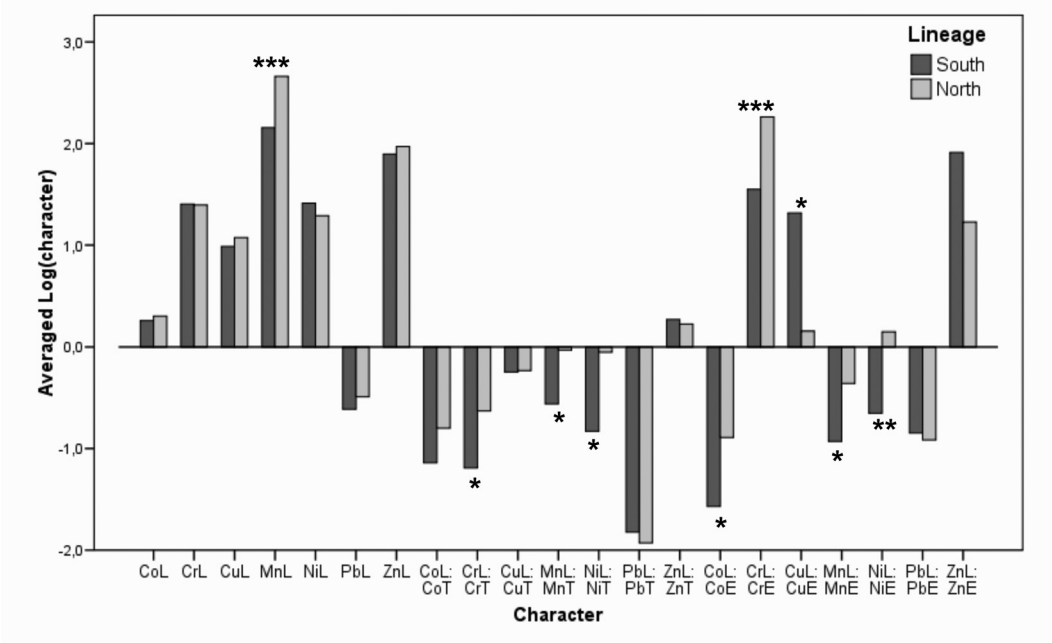

Figure 4.1: ANOVA results for the factor 'Lineage'. Asterisks indicate statistically significant differences. * $P < 0.05$; ** $P<0.01$; *** $P<0.001$. Means for each group were log-transformed for graphical purposes.

4.2.4 Statistical analyses

In a previous study we obtained the total soil contents (CoT, CrT, CuT, MnT, NiT, PbT, ZnT) and extractable soil contents (CoE, CrE, CuE, MnE, NiE, PbE, ZnE) (Quintela-Sabarís *et al.* 2010) (Soil data are available in Supplementary Material S 3.1).

To test whether metal contents in plants differed between plant populations according to their chloroplast lineage and their type (metallicolous or non-metallicolous), a nested analysis of variance (nANOVA) type III SS was performed on the basis of the leaf contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn. These variables were represented as CoL, CrL, CuL, MnL, NiL, PbL and ZnL.

In addition to these 7 variables, the ratio of the metal concentration in leaves over the total and the extractable metal concentration in soils was calculated in order to compare metallicolous and nonmetallicolous populations for the amount of accumulation of these heavy metals. Thus, 14 new variables were created, leading to a total of 21 characters.

All variables were transformed using a BOXCOX transformation in order to improve the fit to a normal distribution of the data. In most cases, BOXCOX yielded a value of power parameter λ equal to 0, so most variables were log-transformed.

The nANOVA model was Lineage, Type, interaction Lineage × Type and population within the interaction Lineage

× Type. Lineage ('North' vs. 'South'; see Quintela-Sabarís *et al.* 2010) and Type (Metallicolous vs. Non-Metallicolous) were fixed effects, whereas Population was included as a random effect.

For the ratios of metals in leaves over extractable metals in soils, only the variation among metalliferous soils could be analysed, because of the very low extractable concentrations of heavy metals found in non-metalliferous soils. In these cases, the nANOVA model was Lineage and Population within Lineage.

Moreover, the relationship among metals in leaves and soils was evaluated using the non-parametric Spearman's Correlation Coefficient. We chose this test since it does not require assumptions of normality of data.

A Principal Component Analyses (PCA) was performed on means of leaf metal contents for each population. A Varimax rotation was used in order to make the interpretation of the PCs easier. Through the PCA, we summarized the results of the analysis of variance in order to confirm whether plant populations are grouped according to their lineage or type.

All computations were performed with SPSS 15.0 for windows, except the BOXCOX transformations, for which we used the MINITAB 15.1 software.

4.3 Results

The mean contents for each metal in each population are presented in Table 4.2. Except for Cu, the higher metal contents were quantified in leaves from M populations. The case of Mn is especially interesting, given the fact that 37% of plants analysed showed more than 500 $\mu g.g^{-1}$, and 16% more than 1000 $\mu g.g^{-1}$, almost reaching 4000 $\mu g.g^{-1}$ in one plant from population ECO. In addition, 59 plants (30% of total sample) have Zn contents above 100 $\mu g.g^{-1}$.

The Spearman correlation coefficient revealed that for two metals (Co and Mn) the concentrations in leaves are not related to the concentrations in soils (neither total nor extractable) (Table 4.3). The contents of Cr, Ni and Pb in leaves were correlated with total and extractable metals in soils, although the correlation was better with total soil contents than with extractable metals. In the case of Cu and Zn, significant correlations were only found between the metals in leaves and the extractable metals in soils (Table 4.3). CoL and MnL were negatively correlated to soil pH (in acid soils-lower pH-, higher values), whereas NiL was positively correlated to soil pH (at higher pH, higher Ni contents) (Table 4.3).

The ANOVA analyses revealed that, irrespective of the population Type (M or NM), the plants from lineage 'North' showed significant higher values than lineage 'South' for the characters MnL and the ratios CrL:CrT, MnL:MnT, NiL:NiT. In addition, in the case of the ratio leaves:soil extractable, the M populations from lineage 'North' showed higher values of CoL:CoE, CrL:CrE, MnL:MnE and NiL:NiE than M populations from lineage 'South'; whereas CuL:CuE was significantly higher in M populations from lineage 'South' (Fig. 4.1).

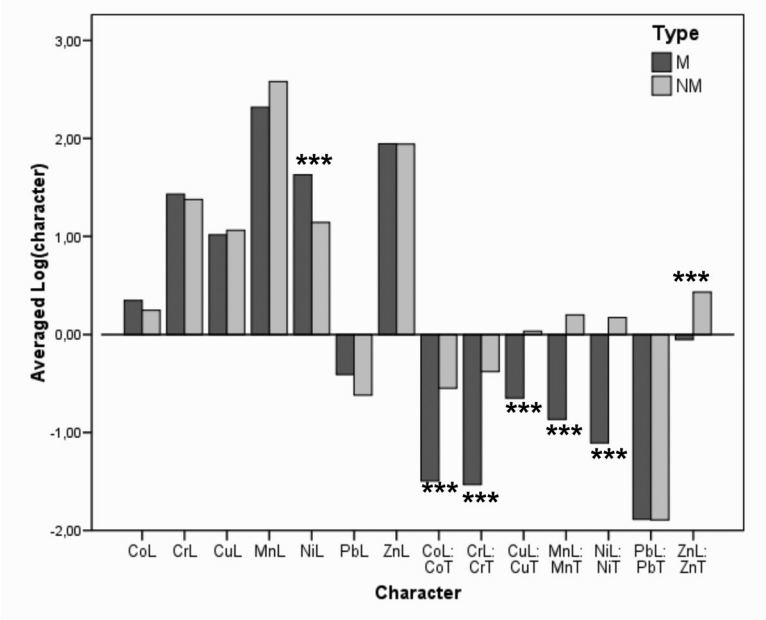

Figure 4.2: ANOVA results for the factor 'Type'. Asterisks indicate statistically significant differences. * $P < 0.05$; ** $P<0.01$; *** $P<0.001$. Means for each group were log-transformed for graphical purposes.

Taking into account the factor Type, significant differences between M and NM populations of Ni content in leaves were found (Fig. 4.2). In addition, the ratios leaf: soil total for all the metals, except Pb, were significantly higher in NM populations.

The interaction Lineage × Type, revealed significant differences only for the characters ZnL and CoL:CoT (Fig. 4.3). ZnL in M populations of lineage 'North' was higher than NM populations from that lineage, whereas in lineage 'South' the M populations had a ZnL value lower than NM ones.

In the case of CoL:CoT, the group of M populations have lower values than NM populations for both lineages.

However, the accumulation of metals varied considerably degree among populations, since for all the characters analysed the factor 'Population' nested within the interaction Lineage × Type showed significant values (in CrL, $P < 0.01$; in the remaining characters, $P < 0.001$).

The combined analysis of the quantities of metals in leaves resulted in a PCA where the M populations from human-polluted sites are placed next to other NM populations along PC1 (which is mainly made up of ZnL, CuL and CoL), whereas the M populations from ultramafic areas are placed apart from the other populations, with negative scores on the first PC and positive scores on the second PC (mainly made up of CrL and NiL) (Fig. 4.4a).

When considering all the ratios leaf: total soil, M populations (with ratio values usually lower than, or near, 1) are grouped in the negative scores on PC1. In contrast, NM populations are more scat-

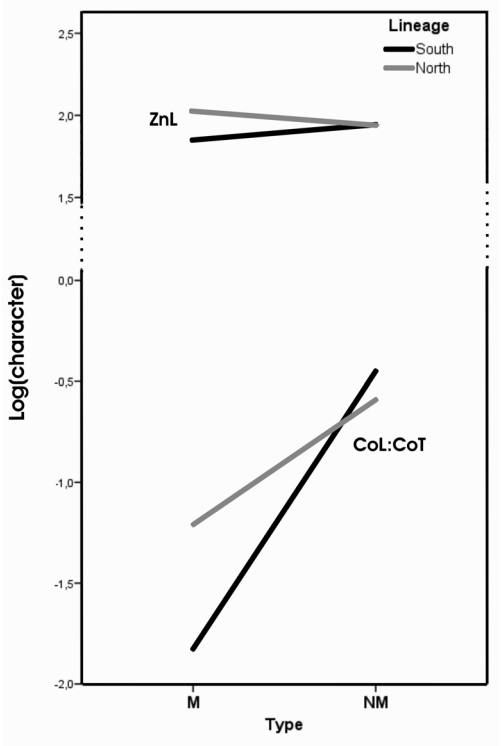

Figure 4.3: ANOVA results for the interaction 'Lineage × Type'. Only traits that showed significant differences among groups were presented. In both cases, $P < 0.05$. Means for each group were log-transformed for graphical purposes.

tered along the two axes (Fig. 4.4b).

In both cases, due to its greater diversity (ultramafic areas and also human polluted sites) M populations from lineage North are more dispersed along the graphics than M populations from lineage South (serpentine outcrops only).

4.4 Discussion

In this work we report the results of a broad scale analysis of the pseudometallophytic shrub species *Cistus ladanifer*. We tried to integrate phylogeographic information about this plant into the analysis of its patterns of heavy metal accumulation in leaves.

We have found that this plant accumulates quantities of metals that in some cases(such as Cr, Mn, Ni, Zn) are above the described critical concentrations in plants (Kabata-Pendias 2001) that is, *C. ladanifer* stores, on average, potentially toxic contents of Cr, Mn and Ni in its leaves. Moreover, the levels of Zn in one third of plants analysed are higher than critical concentrations.

Our data fall within the range of results obtained in previous studies of *C. ladanifer* at local and regional scales (Pratas 1996, Alados *et al.* 1999, Ater *et al.* 2000, Batista 2003, Alvarenga *et al.* 2004, Freitas *et al.* 2004, Díez-Lázaro *et al.* 2006), but for the metals Co, Mn and Ni we have quantified values in leaves that exceed (and in the case of Mn and Ni even double) values reported in the literature

However, our sampling took place in mid summer, after the maximum litterfall of *C. ladanifer* (before summer drought, Núñez-Olivera *et al.* 1993) so the differences between our data and others can be justified by seasonal variations in growth and relative metal contents. Along these lines, the maximum quantities of Sb of *C. ladanifer* were measured in autumn-collected leaves (Murciego Murciego *et al.* 2007).

Through ANOVA analyses, we have found significant effects of the factors Lineage, Type, and Lineage × Type for the quantities of the metals Mn, Ni and Zn, respectively.

All these three elements are micronutrients essential to plant metabolism (Rengel 2004, Epstein and Bloom 2005, and references therein), although their behaviour and functions are different. The foliar concentration of Ni was correlated with soil concentrations. Thus, the M populations (the majority of which are growing in Ni-rich serpentine outcrops) have significantly more foliar Ni than NM ones. This is the general situation in other plant species (Brooks 1987: pp 38).

In the case of foliar Mn, the lineage 'North' possessed significantly higher foliar contents than lineage 'South'. With our data, this difference can not be explained by metal contents in soils (total or available), although a low but significant correlation was observed between MnL and soil pH. However, it seems that soil pH alone cannot explain the higher MnL in lineage North, since ANOVA analyses showed no significant differences in soil pH between lineages (data not shown).

In their work with populations of *C. ladanifer* growing on and around a mine tailing, Alvarenga *et al.* (1999) suggested a relationship between Mn accumulation in leaves and intensity and duration of exposition to sunshine. Due to the broad scale of our work, we do not have evidence to support this suggestion. However, high contents of Mn were observed in populations from the C of the Iberian Peninsula (Lineage 'North'), an area with high summer temperatures, so more investigation would be necessary in order to clarify this topic.

The foliar levels of Zn are influenced by the interaction of factors Lineage and Type. The NM populations of both lineages showed similar levels of foliar Zn, whereas the foliar Zn is higher in M populations from lineage 'North' than M populations from lineage 'South'. This interaction could be caused by the fact that, in our work, human polluted sites (traffic, mine tailings), which presented the higher quantities of Zn both in leaves and soils, are found only in lineage 'North', whereas in lineage 'South' the M populations are all from serpentine outcrops with lower Zn contents.

Baker (1981) defined three basic strategies of metal uptake by plants. Following these definitions, which are based on the ratio between aerial plant part over total metal content in soil, we have observed that *C. ladanifer* behaves as a excluder of Co, Cr and Pb (ratios leaves: soil lower than 1 irrespectively of population type or chloroplast lineage), whereas the accumulation rates of Cu, Mn, Ni and Zn in leaves differs significantly between Lineages and also between population Types.

More specifically, NM populations usually had higher accumulation rates than M ones, under the conditions of our study (growing in non-metalliferous soils). Given this fact, the total amounts of heavy metals (except Ni) in leaves were similar in both types of populations. This difference in accumulation was observed in other pseudometallophytes such as *Arrhenatherum elatius* (Deram *et al.* 2007), *Silene vulgaris* (Chardonnens *et al.* 1998) and the hyperaccumulator *Arabidopsis halleri* (Bert *et al.* 2000), and even in a

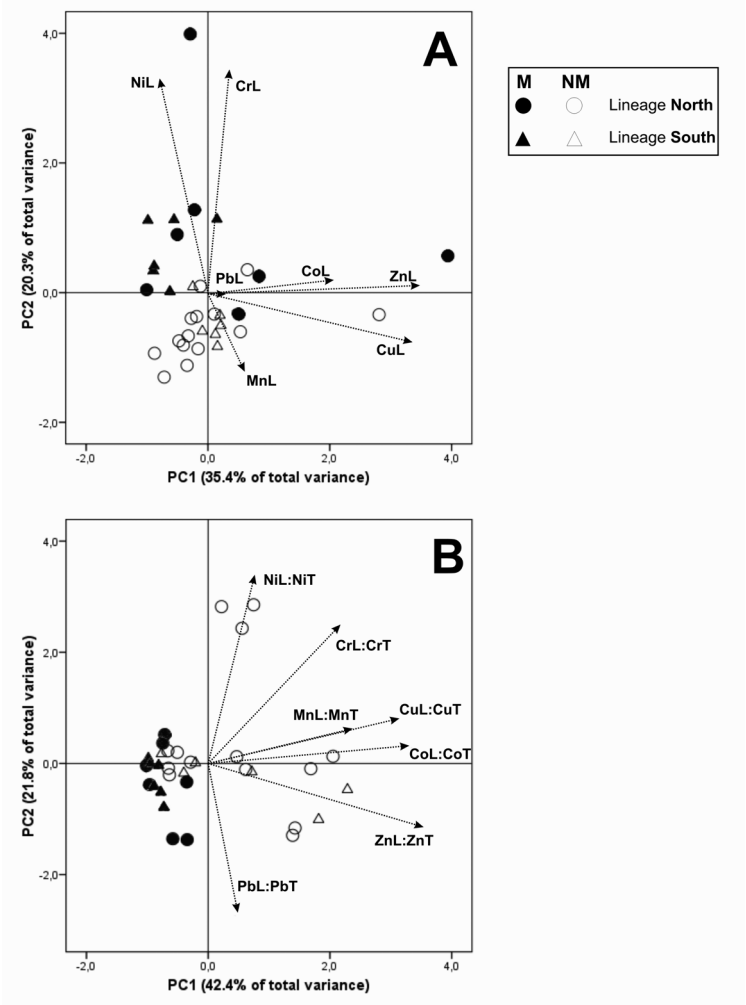

Figure 4.4: Principal Component Analyses (PCA) based on **A**: leaf contents (averaged for each population) of Co, Cr, Cu, Mn, Ni, Pb and Zn; **B**: ratios of leaf and Total soil contents of Co, Cr, Cu, Mn, Ni, Pb and Zn (averaged for each population).
In both cases, filled symbols indicate M populations, whereas empty symbols indicate NM populations. Arrows indicate the contribution of each variable to each of the Principal Components.

Table 4.3: Relationship of metals in *C. ladanifer* leaves with metals in soils and pH. Data show the values of the non-parametric Spearman rho correlation coefficient. Only significant correlations ($P < 0.05$) are presented.

		Metal in Leaves						
		Co	**Cr**	**Cu**	**Mn**	**Ni**	**Pb**	**Zn**
Metal in Soils	**Total**		0.24***			0.73***	0.28***	
	Extractable		0.21**	0.30***		0.70***	0.18*	0.31***
pH		-0.34***			-0.31***	0.20**		

* P<0.05; ** P<0.01; *** P<0.001

work with populations of *Cistus ladanifer* from NE Portugal (Díez-Lázaro *et al.* 2006, Kidd *et al.* 2004). However, most of these studies were all based in hydroponic cultures with controlled metal contents, so we have to be cautious when comparing our results with those.

Deram *et al.* (2007) proposed two explanations for the higher accumulation in NM populations. On the one side, NM populations could develop heavy metal active cellular mechanisms involved in the detoxification of heavy metals. On the other side, M populations could restrict better metal accumulation in aerial parts. Kidd *et al.* (2004) found that NM populations of *C. ladanifer* with low tolerance to Co accumulate more than 3,000 $\mu g.g^{-1}$ Co in shoots but they showed toxicity symptoms when subjected to a 500µM Co treatment; in contrast, the populations with more tolerance to Cu or Ni in hydroponic cultures also showed a low accumulation in aerial parts. Therefore, it appears that in the case of *C. ladanifer*, the most suitable explanation is that of restricted metal accumulation in M populations.

However, there were also differences among M populations. If we consider the ratios computed with extractable metals, which should be more realistically related to plant accumulation abilities, we found that M populations from lineage 'North' showed greater accumulation rates than 'South' for the metals Co, Cr, Mn and specially Ni; whereas M populations from lineage 'South' possessed a greater accumulation rate for Cu.

This phenotypic diversity can be viewed as a result of multiple and independent events of colonisation in areas with heavy metals within diverging chloroplast lineages (Quintela-Sabarís *et al.* 2010). Similarly, serpentine populations of *Cerastium alpinum*, which originated within different recolonisation lineages, developed different growth responses to Ni and Mg (Nyberg-Berglund *et al.* 2004).

The ideal plant species for use in phytostabilisation procedures in an area like the Mediterranean region should be a native plant tolerant to both drought and metal stress, and with an extensive root system. In addition, this species should not accumulate metals into above-ground tissues, in order to prevent further transfer into the food chain and thus reduce human or animal access to contaminants (Frérot *et al.* 2006).

Given these requirements and the ample phenotypic diversity we have observed in *Cistus ladanifer*, Metallicolous populations from lineage 'South' are the best suited for use in phytostabilization procedures in soils polluted with Co, Cr, Mn or Ni, whereas the M populations from lineage 'North' are better for use in Cu polluted soils. However, the considerable differences in the response to those metals among populations means that any remediation procedure should be preceded by a survey that allows the characterization of local ecotypes of *Cistus ladanifer* in relation to heavy metals.

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Chapter 5

Effect of population type and chloroplast lineage on the response of *Cistus ladanifer* L. (Cistaceae) to metals: a hydroponic culture analysis

Previous page: Hydroponically-grown plantlets from different populations of *Cistus ladanifer*. They have been just transferred to plastic buckets in order to perform tests of tolerance to heavy metals. (Photo: C. Quintela-Sabarís)

CHAPTER 5

Effect of population type and chloroplast lineage on the response of *Cistus ladanifer* (Cistaceae) to metals: a hydroponic culture analysis

Keywords: Chlorophyll fluorescence, *Cistus ladanifer*, growth rates, heavy metals, hydroponic culture, tolerance

ABSTRACT

Cistus ladanifer is a pseudometallophyte plant whose metallicolous (M) populations belong to two diverging chloroplast lineages.

Previous studies showed that tolerance to heavy metals could be a characteristic of this plant species. However, it is possible that there are differences in tolerance to metals between population types and/or chloroplast lineages. In order to check these issues, twenty five populations of this species were grown in hydroponic cultures and their tolerance to the trace metals Co, Ni and Zn was assessed.

Growth rates, dry weights and photosystem II fluorescence were measured and transformed into measures of relative tolerance. These data were compared using nested Analysis of Variance and correlation analyses.

Co caused reduction in root growth, whereas Ni and Zn influenced root and shoot growth and also photosynthetic performance. In contrast, the relative increase in the number of leaves was not affected by any treatment. In most cases, the tolerance to metals was similar between population types and chloroplast lineages.

This fact indicates that tolerance to heavy metals could be a common trait in this species. Finally, in view of our findings, we suggest interesting lines for future research, such as the preadaptation of *C. ladanifer* to metals or the possible role of root symbioses in tolerance.

5.1 Introduction

Heavy metals have toxic effects on plant metabolism, such as the production of reactive oxygen species, the blocking of essential functional groups in biomolecules and the displacement of essential metal ions (Schutzendubel and Polle 2002), which subsequently produce reduction in growth, variation in photosynthesis or even death.

Due to these toxic effects, soils with high contents of these elements, either of natural or human origin, are areas with low vegetation cover and, in the case of human-polluted sites, can constitute environmental problems due to the leaching of metals into groundwater or the migration of contaminated soil by erosion and/or dispersal by wind (Ruttens *et al.* 2006). Some plant species have developed mechanisms for metal tolerance (e.g. chelation of metals in the cytosol by phytochelatins, (Goldsbrough 2000)) whose objective is to avoid the build-up of toxic concentrations at sensitive sites within the plant cell (Hall 2002).

This tolerance can be ‘constitutive’ (=’species-wide’; i.e. common to all the individuals of a given species) or ‘population-specific’ (i.e. the tolerance to heavy metals have evolved only in certain

populations of a given species) (Pollard *et al.* 2002).

Different research has indicated that tolerance to heavy-metals in plants; i.e., the ability to grow and survive in soils toxic to other plants, is a characteristic governed by a low number of major-genes, which are also modified by a large number of minor-genes (reviewed in Macnair *et al.* (2000)). As a consequence of the genetic determination of this characteristic, a variation in responses to heavy metals is expected and observed within different species (e.g. Zn tolerance in *Arabidopsis halleri*, Pauwels *et al.* (2006)). This variation in responses is clearer in those species whose metallicolous populations have evolved independently, as shown in *Silene paradoxa* (Gonnelli *et al.* 2001), *Cerastium alpinum* (Berglund *et al.* 2004) or *Thlaspi caerulescens* (Assunção *et al.* 2003).

Cistus ladanifer L. (Cistaceae) is a woody, semideciduous shrub from the Western Mediterranean Area (from the South of France to North of Morocco and Algeria) (Demoly and Montserrat 1993). This plant is a pseudometallophyte; thus, it is present in non-metalliferous soils (mainly derived from acid rocks) and it also has colonised metalliferous areas (serpentine outcrops and mine tailings) throughout its distribution area (Alvarenga *et al.* 2004, Ater *et al.* 2000, Batista 2003, Díez Lázaro *et al.* 2006, Freitas *et al.* 2004, Pratas *et al.* 2005). Using cpSSR markers, we have inferred that the metallicolous populations of this species (i.e., those present on metalliferous soils) have arisen within two diverging chloroplast lineages (Quintela-Sabarís *et al.* 2010). On the basis of similar genetic diversity values between metallicolous (M) and non-metallicolous (NM) populations, we proposed that tolerance to heavy metals could be a 'constitutive' character in *C. ladanifer*.

In the present paper, we investigated the tolerance of *C. ladanifer* to the following heavy metals: Co, Ni and Zn, in 25 populations throughout its whole range of distribution. Previous work has assessed the tolerance of *C. ladanifer* to different heavy metals, but only in five populations from NE of Potugal (Kidd *et al.* 2004). Here, we asked whether tolerance to heavy metals is present both in M and NM populations of *C. ladanifer* and whether differences exist in tolerance to these metals between population types (metallicolous vs. non-metallicolous) and/or chloroplast lineages. Specifically, we estimated tolerance by measuring growth, biomass and photochemical efficiency.

5.2 Material and methods

5.2.1 Populations studied

Twenty-five populations of *C. ladanifer* sampled throughout its natural geographic distribution area were included in this work. These populations were classified in two 'soil types': Metallicolous (M) and Non-metallicolous (NM) (see supplementary material S 3.1 and S 5.1), based on previous soil analyses (Quintela-Sabarís *et al.* 2010). M populations include plants growing in serpentine outcrops from Morocco, Portugal and Spain and also populations developed on mine tailings from

Portugal and Spain.

In addition, previous analyses of chloroplast microsatellites (cpSSR) revealed that these populations come from two chloroplast lineages that spread independently throughout the N of Morocco and SE of the Iberian Peninsula ('South' lineage) and the rest of the Iberian Peninsula ('North' lineage) after the Last Glacial Maximum (Quintela-Sabarís *et al.* 2010). (Table 5.1).

In each population, a longitudinal transect was established at random. 10 plants separated by at least five meters were selected along this transect and their ripe fruits were collected.

5.2.2 Plant material and growth conditions

Samples of seeds from each population were subjected to a dry-heating treatment (100ºC for 30 minutes) in order to break the physical dormancy and increase germination percentages (Perez-Garcia 1997). The treated seeds were then sown in Petri dishes with sterilized acid-washed sand and moisturized with distilled water. Seeds were placed in a culture chamber under the following conditions: 16/8 h light/darkness, day/night temperature 20/15 ºC, RH 70%, and PPFD 190 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Four weeks after germination (when the plantlets reached a four-leaf stage), they were carefully transferred to polystyrene sheets suspended in a nutritive solution with low concentrations of trace metals (control solution) at pH 4.5. This solution, optimized for *Cistus ladanifer* by Kidd *et al.* (2004), had the following composition (µM): 2000 $CaCl_2.2H_2O$, 1000 $MgSO_4.7H_2O$, 250 NH_4NO_3, 50 KH_2PO_4, 200 NaOH, 150 KCl, 25 $ZnSO_4.7H_2O$, 20 $MnSO_4.H_2O$, 15 $CuSO_4.5H_2O$, 15 FeEDTA, 10 H_3BO_3, 0.0143 $(NH_4)_6Mo_7O_{24}.4H_2O$.

Stock solutions of 100-(macronutrients) and 1000-strength (micronutrients) were made up and diluted appropriately. Culture solutions were continuously aerated with an aquarium air pump and changed every week. After 42 d in this solution, seedlings of similar size were selected and randomly placed into thirty 6 L-plastic buckets, each holding 10 seedlings. At this stage seedlings were transferred to a greenhouse (20–28 ºC) with natural light, where they continued to grow until the end of the experiment.

5.2.3 Treatment with metals and ecophysiologycal measurements

Due to space limitations, the 25 populations were grouped in batches of five populations each. A total of 10 successive experiments (two for each batch) were carried out, replicating the same growth conditions previously described.

In each batch, five buckets of *C. ladanifer* plants (each containing two replicates for each population) were randomly allocated to one of the following treatments with trace metals: cobalt (Co), nickel (Ni) and zinc (Zn), and three controls. Treatment concentrations and chemical form in which the metal was added were chosen following Kidd *et al.* (2004) (see Table 5.2 for details). Solutions were completely replaced every three days to

Table 5.1: List of *Cistus ladanifer* populations included in our analyses. For each population, the lineage (North, South) and the type (M, NM) are indicated.

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with dispersed *Pinus pinea* and *P. halepensis* trees	4.79º W	36.84º N
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks	4.64º W	34.95º N
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89º W	35.29º N
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18º W	37.88º N
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87º W	41.85º N
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78º W	37.07º N
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03º W	37.39º N
Martinchel (PMA)	North	NM	Sedimentary material (gravels, clays)	Dense *Pinus pinaster* plantation	8.29º W	39.52º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

Table 5.1: (continued)

Population (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82º W	41.52º N
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75º W	41.78º N
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98º W	37.03º N
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29º W	40.43º N

Chloroplast lineage and population type following Quintela-Sabarís *et al.* (2010). Longitude and latitude are expressed in decimal degrees.

maintain nutrient supply and treatment metal concentrations.

Length of the longest root (L_R), shoot length (L_S) and the number of leaves (N_L) were recorded before plants experienced trace metals (abbreviated as L_{R0}, L_{S0}, N_{L0}) and at harvesting, after 14 days of growth under the different treatments (abbreviated as L_{R14}, L_{S14}, N_{L14}). We obtained three growth measurements (R_E, S_E, N_L) as the differences between variables on days 0 and 14. In addition, harvested plants were dried at 60ºC to constant weight and the dry weight (abbreviated as DW) was measured.

Photochemical efficiency, as estimated by chlorophyll fluorescence, was measured in vivo on three fully expanded leaves at the top of each plant the night before harvesting (day 13), using a pulse-amplitude modulated fluorometer (Mini-Pam, Walz, Effeltrich, Germany) in 15 of the 25 populations included in this study. Measuring light and saturating light pulses (>4000 µmol photons$\cdot m^{-2}\cdot s^{-1}$, 0.8 s pulse length, actinic white light) were applied through a fibre optic probe at a 60º angle relative to the sample and 12 mm from the leaf. The maximum quantum yield of photosystem II (PSII) was assessed from the ratio $F_v/F_m = (F_m - F_0)/F_m$ (see Bolhàr-Nordenkampf *et al.* 1989), where F_0 and F_m are defined as minimal and maximal fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open. This parameter was measured at night, with dark-adapted plants to ensure that all their PSII reaction centres were open. The maximum quantum yield esti-

Table 5.2: Summary of metal treatments and the chemical form in which they were added to nutrient solutions.

Trace metal	Chemical form added	Treatments (μM)			
		Control	Co	Ni	Zn
Co	$CoSO_4.7H_2O$	0	100	0	0
Ni	$NiSO_4.6H_2O$	0	0	250	0
Zn	$ZnSO_4.7H_2O$	25	25	25	250

mates the efficiency of excitation energy capture by open PSII reaction centres (Butler and Kitajima 1975).

In order to eliminate most of variation on responses unrelated to metal treatments, both growth (R_E, S_E, N_L) biomass (DW) and photochemical efficiency measurements were transformed as a percentage of control plants = 100 × (response in metal treatment) / (mean value of the response in the control), following the original proposal of Wilkings (1978) for root elongation.

Thus, we finally obtained five relative measures of metal-tolerance: relative root elongation (R_{RE}), relative shoot elongation (R_{SE}), relative increase of number of leaves (R_{NL}), relative dry weight (R_{DW}) and relative Yield (R_Y).

5.2.4 Statistical analyses

For each metal (Co, Ni and Zn), we tested for differences between metal treatment (metal vs. control), population type (metallicolous vs. non-metallicolous), chloroplast lineages (North vs. South) and among plant populations, on R_{RE}, R_{SE}, R_{NL}, R_{DW} and R_Y. We used a nested analysis of variance (nANOVA) in which metal treatment (Treatment), population type (Type) and chloroplast lineage (Lineage) were fixed factors, and population was a random factor nested within the interaction Lineage×Type. In order to extract the effects of initial plant size from the analysis, the measures L_{R0}, L_{S0} and N_{L0} were introduced in the model as covariates.

Before carrying out statistical analyses, the data were explored in order to i) detect extreme values, which were not included in the analyses; and ii) check the homogeneity of variances and the normality of the data. When necessary, data were transformed in order to meet ANOVA assumptions of homogeneity and normality.

Finally, the relationships between tolerance to each metal and the previous exposition to metals in field were examined through the computing of the non-parametric Spearman's correlation index among R_{RE}, R_{SE}, R_{NL}, R_{DW}, R_Y and the total (CoT, NiT, ZnT) and extractable (CoE, NiE, ZnE) contents of Co, Ni and Zn in the soils from the populations of origin.

Data analyses were conducted in SPSS (v.15, SPSS Inc., Chicago, IL, USA).

5.3 Results

5.3.1 Effects of 'Treatment'

The three metal treatments caused significantly lower R_{RE} values than the controls (*P*: $R_{RE\text{-}Co}$ 0.001, $R_{RE\text{-}Ni} < 0.001$, $R_{RE\text{-}Zn} < 0.001$), whereas R_{NL} showed no differences between metals and controls. For the other three variables, only plants treated with Ni or Zn showed lower values than controls (Fig. 5.1).

5.3.2 Effects of the interaction 'Treatment × Type'

The three variables related to growth (R_{RE}, R_{SE} and R_{NL}) showed no significant differences for the interaction Treatment × Type (*P*: $R_{RE\text{-}Co}$ 0.299, $R_{RE\text{-}Ni}$ 0.974, $R_{RE\text{-}Zn}$ 0.654, $R_{SE\text{-}Co}$ 0.803, $R_{SE\text{-}Ni}$ 0.479, $R_{SE\text{-}Zn}$ 0.321, $R_{NL\text{-}Co}$ 0.207, $R_{NL\text{-}Ni}$ 0.902, $R_{NL\text{-}Zn}$ 0.852). That is, M and NM populations showed the same growth responses to each of the treatments (Fig. 5.2). In contrast, R_Y revealed significant differences in the treatments with Ni and Zn: whereas 'Controls' from M and NM populations showed similar R_Y values, NM populations showed lower yield values than M populations when subjected to treatments with metals (Fig. 5.2; *P*: $R_{Y\text{-}Co}$ 0.461, $R_{Y\text{-}Ni}$ 0.006, $R_{Y\text{-}Zn}$ 0.009).

In addition, R_{DW} revealed significantly different responses in M and NM populations to the treatments 'Control' and 'Zn'. However, the mean for 'Control' plants (either M or NM) was higher than mean values for 'Zn' plants (Fig. 5.2; *P*: $R_{DW\text{-}Co}$ 0.434, $R_{DW\text{-}Ni}$ 0.186, $R_{DW\text{-}Zn}$ 0.010).

5.3.3 Effects of the interaction 'Treatment × Lineage'

When considering the chloroplast lineages, we only found significant differences for the metals Ni and Zn (Fig. 5.3). Plants from lineage 'North' treated with Ni showed a greater reduction in R_{RE} than plants from lineage 'South' (Fig. 5.3; *P*: $R_{RE\text{-}Co}$ 0.851, $R_{RE\text{-}Ni}$ 0.033, $R_{RE\text{-}Zn}$ 0.533). Plants from lineage 'North' had values of R_{DW} similar to the 'controls', whereas the mean R_{DW} of plants from lineage 'South' was around 60% (Fig. 5.3; *P*: $R_{DW\text{-}Co}$ 0.162, $R_{DW\text{-}Ni}$ 0.753, $R_{DW\text{-}Zn}$ 0.021).

In the case of R_Y, 'controls' of lineage 'South' were higher than 'controls' of lineage 'North', whereas metal-treated plants from lineage 'North' were higher than metal-treated plants from lineage

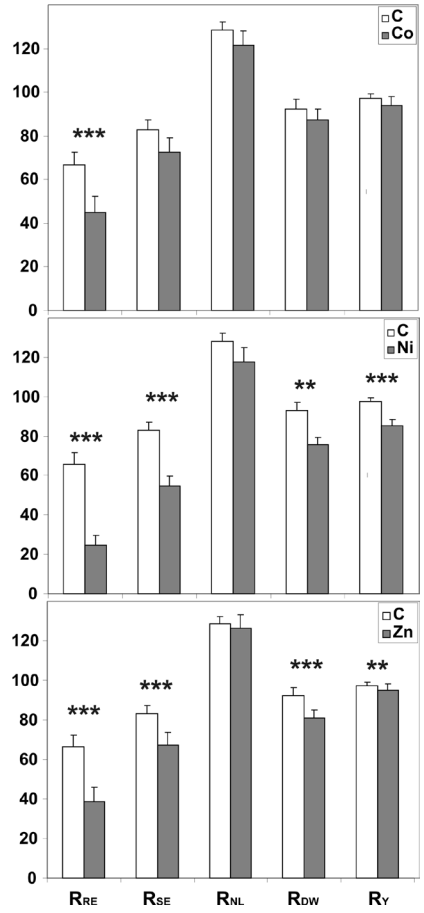

Figure 5.1: nANOVA results for the factor 'Treatment'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001

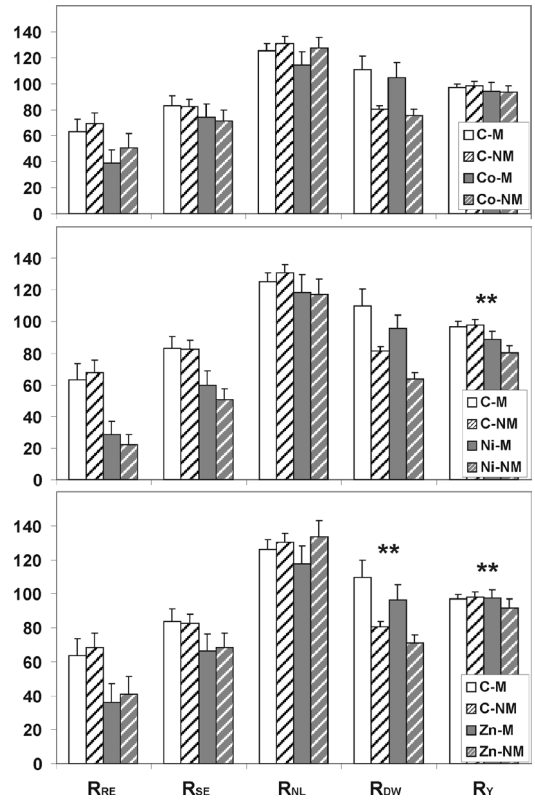

Figure 5.2: nANOVA results for the factor 'Treatment × Type'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

'South'. In other words, when subjected to metal treatments (Ni or Zn) plants from lineage 'South' suffered a greater decrease in their yield than plants from lineage 'North' did (Fig. 5.3; *P*: $R_{Y\text{-}Co}$ 0.941, $R_{Y\text{-}Ni}$ 0.007, $R_{Y\text{-}Zn}$ 0.037).

5.3.4 Other interactions

The interaction 'Treatment × Lineage × Type' was significant only in the case of RDW for the metal Zn (F= 5.56, P = 0.023).

The interaction 'Treatment × Pop(Lineage × Type)', which reflects differences in response to treatments among populations, showed significant differences in the case of the metal Ni for the variables R_{RE} (P = 0.018), R_{SE} (P = 0.049) and R_{DW} ($P < 0.001$).

5.3.5 Correlation tolerance-metals in soils

The values of the Spearman correlation index were non-significant for all the comparisons among metals and tolerance indices (data not shown). Thus, populations from soils with low metal contents show similar (or even higher) tolerance indices than populations from metalliferous areas (Fig. 5.4).

5.4 Discussion

Our work is the first broad-scale assessment of the tolerance of the pseudometallophyte shrub *Cistus ladanifer* to the metals Co, Ni and Zn.

We have shown that each metal caused different toxic effects: Ni and Zn treatments affected root and shoot growth, biomass and photochemical efficiency, Co merely reduced root growth, whereas the number of leaves were not affected by any treatment. Similarly, Kidd *et al.* (2004) also found average reduction in relative root elongation in five populations of *C. ladanifer*, and a greater effect for Ni and Zn than for Co.

Although these three metals have been described in the bibliography as pos-

sessing different critical toxic concentrations (e.g. Kabata-Pendias 2001), we can explain the different effects for each metal on the basis of previous analyses of heavy-metal accumulation that we performed in field-collected leaves from the same populations (data as yet unpublished): we observed that *C. ladanifer* clearly excludes Co and, depending on the chloroplast lineage or the population type, this species behaves as an excluder or an accumulator (*sensu* Baker 1981) of Ni and Zn.

Thus, due to the different behaviour of each metal, we expect Co effects only on roots, as observed, since these organs are the only parts in direct contact with the metal (the plant excludes its transport to aerial parts); however, Ni and Zn can be transported to and accumulated into aerial parts, which may produce alterations of shoot growth and photochemical efficiency, as we indeed detected.

Given the diverse parameters employed by researchers to measure tolerance to heavy metals (growth, survival rates, biomolecules,… for a review see Köhl and Lösch (2004)), and taking into account the previous statement, for future analysis of metal tolerance in plants it would be useful to know the strategy of response to heavy metals in a given species in order to determine the best parameter to be measured.

The significant differences observed for the interaction treatment × lineage point to different mechanisms of response to heavy metals in each independent lineage, as was inferred in *Cerastium alpinum* (Nyberg Berglund *et al.* 2004). However, we have to keep in mind that these differences can be influenced by the fact that lineage North includes M populations from ultramafic areas and mine tailings, whereas the M populations from lineage South are only from ultramafic areas. In fact, the interaction treatment × lineage × type was only significant for R_{DW} for the metal Zn.

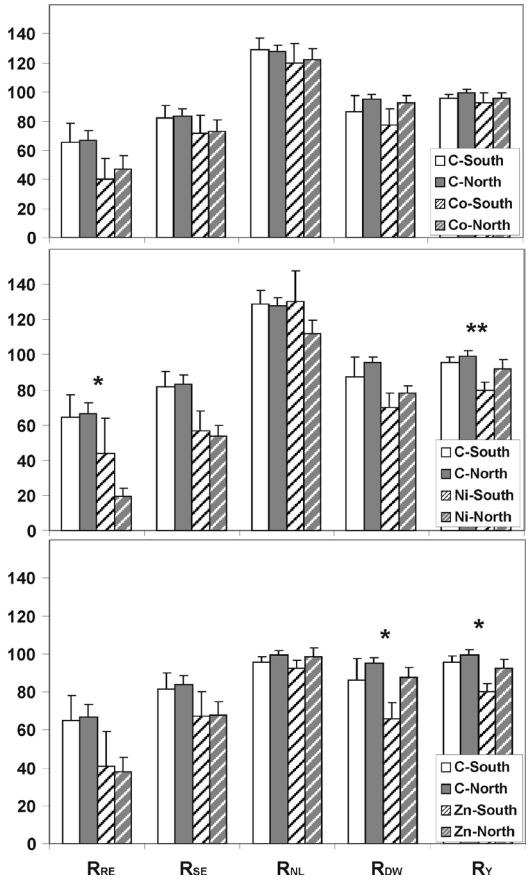

Figure 5.3: nANOVA results for the factor 'Treatment × Lineage'. Bars indicate the value of marginal means for each variable and each group after back-transformations. Each graph refers to one of the trace metals analysed (top- Co; middle- Ni; bottom- Zn). Significant differences are indicated by asterisks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

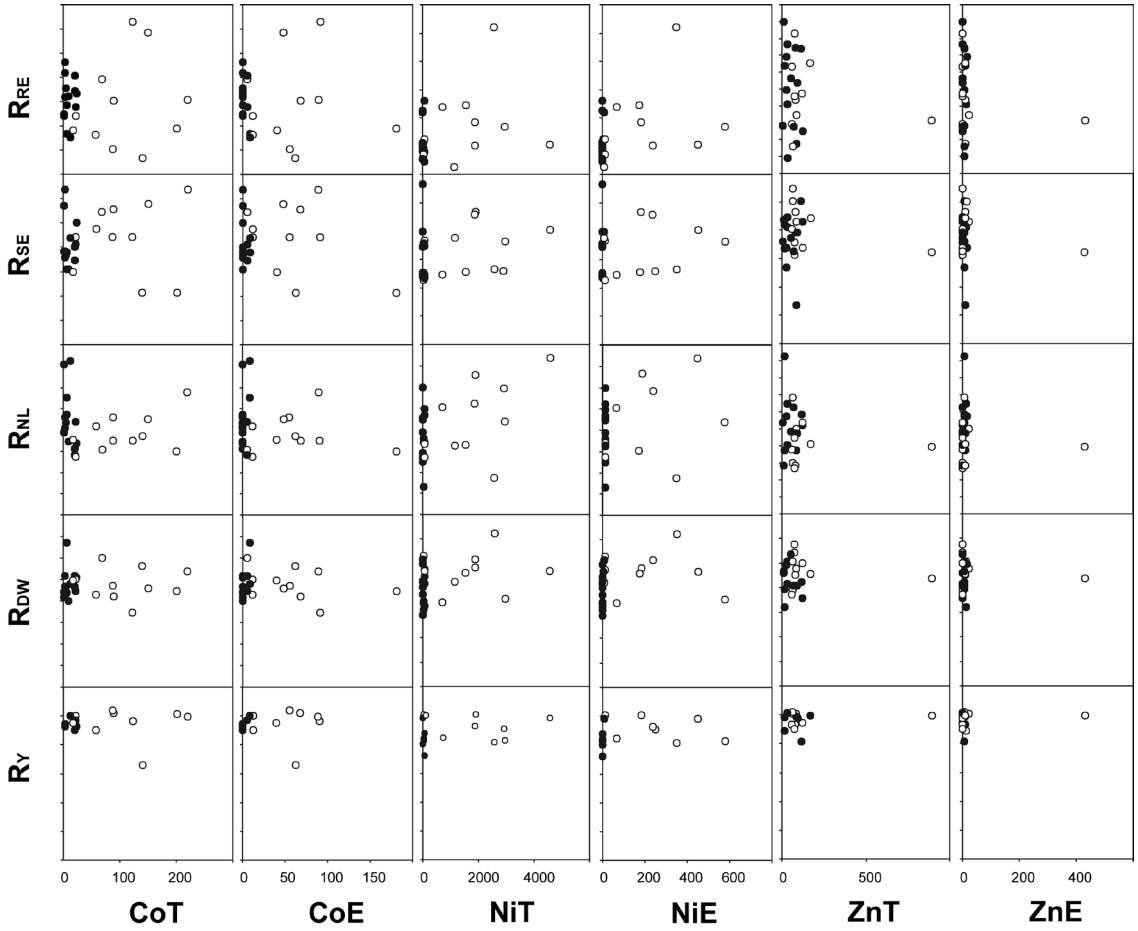

Figure 5.4: Scatterplots of Tolerance Indices *vs.* total (CoT, NiT, ZnT) or Ammonium-acetate/EDTA extractable (CoE, NiE, ZnE) contents of Co, Ni and Zn in the soils of origin. Filled circles indicate NM populations, whereas empty circles indicate M populations.

Based on results previously obtained of the phylogeographic origin and genetic diversity of M populations of *C. ladanifer* (Quintela-Sabarís *et al.* 2010), we proposed tolerance to heavy metals as a constitutive trait in this species. Our results in hydroponic cultures partially support this previous assertion: with the exception of R_{DW} in Zn and R_Y in Ni and Zn, the other measured parameters showed non-significant differences for the interaction treatment × type; whereas the interaction lineage × treatment × type showed significant differences only in the case of R_{DW} and Zn. In addition, populations with low concentrations of metals in the soils of origin showed indices of tolerance similar or even higher than M populations.

We may speculate that findings point to two possible explanations (which are not mutually-exclusive): Firtsly, a preadaptation of *Cistus ladanifer* to soils rich in heavy metals. Macnair (1987) suggested that plant species that are preadapted for any of the harsh conditions of metal rich soils (such as water stress or nutrient shortage) successfully colonize these regions more easily; moreover, Taylor and Levy (2002) detected preadaptation to low Ca:Mg ratios (one of the selective factors of serpentine soils) in a variety of *Phacelia dubia* that was endemic to granite outcrops. In the case of *C. ladanifer*, different authors provide arguments which support the hypothesis of preadaptation: Núñez-Olivera *et al.* (1996) uncovered a high leaf plasticity in *C. ladanifer*, which implies an intrinsic adaptation to scarcity of water and nutrients in this species; Alados *et al.* (1999) suggested that the abilities of *C. ladanifer* inhabiting acidified soils with low Ca concentrations allowed the plants to perform well in serpentine soils in spite of the presence of heavy metals; finally, Kidd *et al.* (2004) observed that plants from non-metallicolous population of *C. ladanifer* maintained high growth rates in presence of Zn in hydroponic cultures.

A second hypothesis that should be explored in future research, partially linked to that of preadaptation, is the possibility that *C. ladanifer* colonised metalliferous areas through collaboration with soil micro-organisms such as fungi and rhizobacteria, given that ectomycorrhizal fungi can provide protection and improve the tolerance to heavy metals in host species (Jentchske *et al.* 2000). Along these lines, Ramos Solano *et al.* (2006) have detected different species of plant-growth promoting rhizobacteria (PGPR) in *C. ladanifer* roots, most of them involved in P-mobilisation and production of siderophores. In addition, more than 30 fungal species have been recorded as establishing symbiotic relations (ectomycorrhiza) with *C. ladanifer* in the literature (Comandini *et al.* 2006). Thus, the negative effects of heavy metals we observed in the plants under metal-treatments could be caused by the lack of these root symbionts in the conditions of hydroponic culture (Epstein and Bloom 2005).

In summary, our investigation revealed that Co, Ni and Zn produced different toxic effects in C. ladanifer plants growing in hydroponics. In most cases the response to metals is similar between population types or chloroplast lineages, a fact that points to the tolerance to heavy metals as a common trait in this species. Moreover, interesting lines for future research, such as the occurrence of preadaptive traits in this species or the role of root symbioses in tolerance to metals, are suggested.

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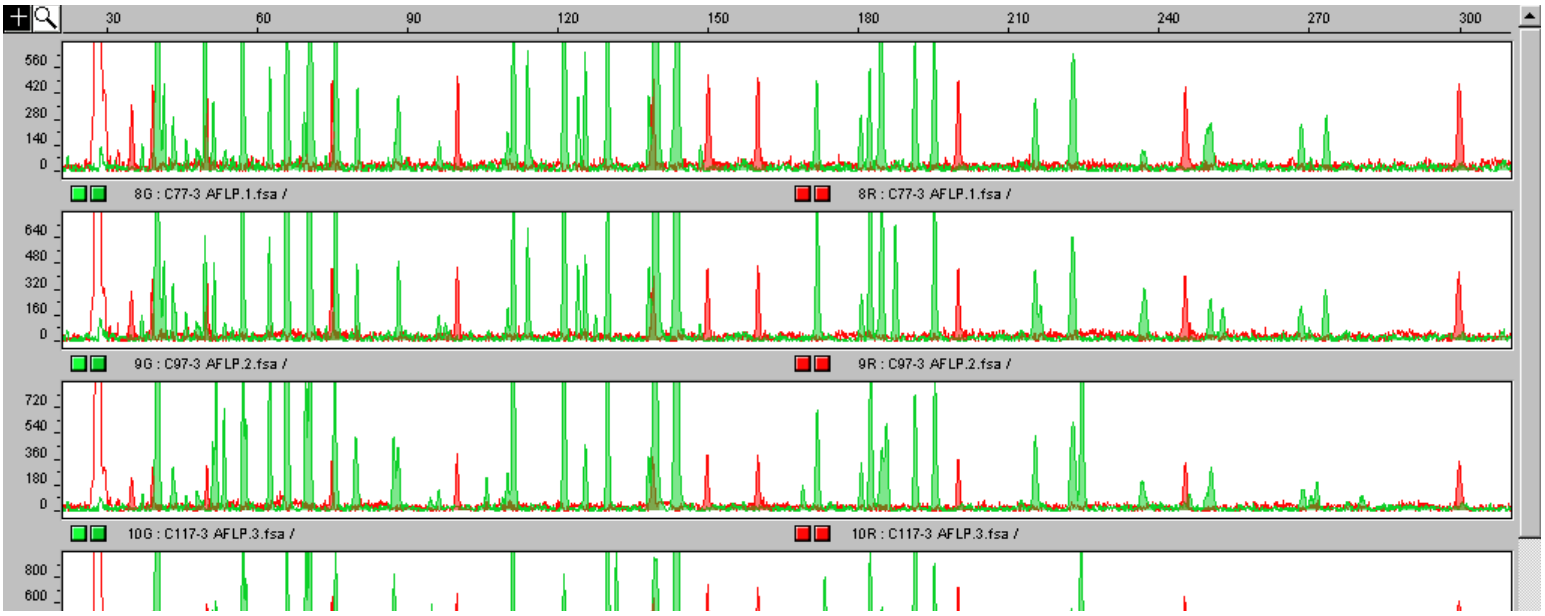
Chapter 6

AFLP analysis of the pseudometallophyte *Cistus ladanifer*: a comparison with cpSSRs and an exploratory genome scan to investigate loci associated to soil variables



Previous page: Screenshot of electropherograms of different *C. ladanifer* plants genotyped with AFLP markers. Red peaks correspond to the molecular weight marker, whereas green peaks indicate loci amplified with the primer pair: NED-EcoRI- AGG/ MseI- CAG. (Image: C. Quintela-Sabarís)

CHAPTER 6

AFLP analysis of the pseudometallophyte *Cistus ladanifer*: a comparison with cpSSRs and an exploratory genome scan to investigate loci associated to soil variables

Keywords: Amplified Fragment Length Polymorphism, chloroplast microsatellites, *Cistus ladanifer*, Generalized Estimating Equations, heavy metals

ABSTRACT

Cistus ladanifer is a pseudometallophyte which grows in different metal-rich substrates from the Western Mediterranean area.

We have analysed 33 populations of this species with AFLPs in order to i) assess the genetic patterns of colonisation of metalliferous areas by *C. ladanifer* obtained with an AFLP genome scan and to compare them with previous cpSSRs results and ii) identify loci potentially linked to tolerance to metalliferous soils.

AFLP results were partially congruent with previous cpSSR results, revealing no influence of soil type on the genetic diversity and differentiation of this species. However, some differences arose, mainly due to different properties (mutation rate, ploidy level, inheritance, …) inherent in each marker.

Then we used Generalized Estimating Equations models to test the correlation between allele distribution and soil data. These regression analyses showed that the total soil contents of Mn has an important effect on allele distribution in *Cistus ladanifer*, the strongest in relation to the other soil variables we also have analysed.

Moreover, we report the detection of a particular allele with a possible role in tolerance to high Mn concentrations in soils.

6.1 Introduction

Soils with high concentrations of metals (metalliferous soils) are toxic to most plants and other living organisms (Shaw *et al.* 2004). Moreover, soils polluted by human activities, particularly those activities related to the production of metals (mine tailings, smelting areas ...) constitute a threat to the environment and public health: they usually have a sparse (or even absent) plant cover and thus metals can leach into groundwater or the polluted soil can be dispersed by wind which then affects productive agricultural land or natural reserves (Ruttens *et al.* 2006, Tordoff *et al.* 2000). However, the metalliferous areas also may be considered as ecological islands in which different metal-tolerant plant species are found. Thus, soils with high contents of heavy metals provide the opportunity to study the establishment and differentiation of plant populations under severe selection pressure (Lefèbvre and Vernet 1990).

Among metal-tolerant species we may distinguish between strict metallophytes (or eumetallophytes), whose populations only grow on metalliferous substrates, and pseudometallophytes (or facultative metallophytes), which can grow both in metalliferous and non-metal-

Table 6.1: Description of *Cistus ladanifer* populations studied.

Pop (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat	N
Sierra de Aguas (EAC)	South	M (u)	Serpentinised peridotite	Open shrubland with scattered *Pinus pinea* and *P. halepensis*	4.79º W	36.84º N	7
Almodóvar (EAL)	North	M (h)	Clays close to a road	Grassland with dispersed *C. ladanifer* plants	5.65º W	36.16º N	8
Benalup (EBE)	South	NM	Sandstone	Cleared *Quercus suber* forest with *Phyllirea angustifolia*, *Calicotome villosa* and *C. ladanifer*	5.73º W	36.32º N	8
Cardeña (ECA)	North	NM	Granite	Open *Quercus ilex* forest with *C. ladanifer* and *Pistacia lentiscus*	4.36º W	38.28º N	9
La Codosera (ECO)	North	M (m)	Sb mine tailing	Shrubland dominated by *C. ladanifer* with *Dittrichia viscosa*	7.08º W	39.19º N	8
Ciudad Rodrigo (ECR)	North	NM	Quartzites	Dense matorral with isolated *Quercus ilex* trees	6.49º W	40.63º N	9
Despeñaperros (EDE)	North	M (h)	Quartzites, close to the highway.	Dense *Cistus ladanifer* shrubland with *Quercus ilex* and *Juniperus oxycedrus*	3.51º W	38.39º N	8
Fuente Saúco (EFS)	North	NM	Sandstone and conglomerates	Shrubland with *Quercus ilex*	5.51º W	41.19º N	7
El Guijo (EGJ)	North	NM	Slates	Open dehesa with *Quercus ilex* and isolated *C. ladanifer* plants	4.77º W	38.52º N	8
Grazalema (EGR)	South	NM	Decarbonated limestone	Shrubland with *C. ladanifer* and *C. monspelliensis*	5.27º W	36.78º N	9
Guadarrama (EGU)	North	NM	Granite	Dense shrubland with isolated *Quercus ilex*	4.10º W	40.68º N	9
Mazagón (EMA)	South	NM	Sand deposits	*Eucalyptus globulus* and *Pinus pinea* plantations	6.84º W	37.15º N	9
Monte Furado (EMF)	North	NM	Schists	Dense shrubland with *Quercus ilex*	7.20º W	42.39º N	8
Ricobayo (ERB)	North	NM	Quartzites and filites	Open shrubland with *Quercus ilex*	5.81º W	41.70º N	5
Sierra Bermeja (ESB)	South	M (u)	Serpentinised peridotite	Dense shrubland with scattered *Pinus pinaster*	5.18º W	36.48º N	9
Sierra Palmitera (ESP)	South	M (u)	Serpentinised peridotite	Open matorral with *Quercus coccifera* and *Ulex* sp.	5.07º W	36.60º N	8
Sierra de Tolox (ETO)	South	M (u)	Serpentinised peridotite	Open matorral with scattered *Pinus pinaster* and *Ulex* sp.	4.93º W	36.68º N	9
Valdecaballeros (EVC)	North	NM	Sedimentary material (gravels, clays)	Dense shrubland with *Pinus pinaster*	5.34º W	39.33º N	7
Bni Hadifa (MBH)	South	NM	Sandstones	Open *Pinus halepensis* forest	4.17º W	35.02º N	8
Bab Tazaa (MBT)	North	NM	Micaschists	Open shrubland with dispersed *Cistus* plants	5.24º W	35.08º N	7
El Jebha (MEJ)	South	NM	Sandstone	Open *Pinus halepensis* forest	4.64º W	35.18º N	9
Ketama (MKE)	South	NM	Schists	Dense shrubland with evergreen oaks and *Cistus laurifolius*	4.64º W	34.95º N	7
West Bni Bouchra (MSI)	South	M (u)	Serpentinised peridotite	Open matorral with *Pistacia lentiscus*, *Phillyrea latifolia* and *Tetraclinis articulata*	4.90º W	35.30º N	8

Lineage and Type were defined on the basis of analyses published in Quintela-Sabarís *et al*. 2010. *Lineage:* cpSSR lineage ('North', 'South'); *Type*: M (metallicolous), NM (non-metallicolous). Geographic coordinates are given in decimal degrees. *N*: number of plants genotyped with AFLP in each population.

Table 6.1: (continued)

Pop (Code)	Lineage	Type	Substratum	Vegetation	Long	Lat	N
East Bni Bouchra (MSII)	South	M (u)	Serpentinised peridotite	Open matorral with *Halimium atriplicifolium*, *Pistacia lentiscus* and *Tetraclinis articulata*	4.89° W	35.29° N	7
Tanger (MTA)	South	NM	Sandstone	Open *Pinus pinaster* forest	5.93° W	35.78° N	8
Aljustrel (PAL)	North	M (m)	Pyrite mine tailing	Dense matorral dominated by *C. ladanifer* and *Lavandula stoechas*	8.18° W	37.88° N	7
Bragança (PBR)	North	M (u)	Dunite	Dense shrubland with *Pinus pinaster* and *Genista* sp.	6.87° W	41.85° N	8
Burgau (PBU)	North	NM	Limestone	Dense shrubland with *Chamaerops humilis* and *Pistacia lentiscus*	8.78° W	37.07° N	8
Corte Figueira (PCF)	North	NM	Schists	*Quercus suber* 'Montado' with dense cover of *Cistus ladanifer*	8.03° W	37.39° N	6
Macedo dos Cavaleiros (PMC)	North	M (u)	Serpentinised peridotite	Dense shrubland with *Quercus ilex*	6.82° W	41.52° N	6
Samil (PSA)	North	M (u)	Serpentinised peridotite	Open matorral with *Alyssum serpyllifolium* and *Quercus ilex*	6.75° W	41.78° N	7
São Vicente (PSV)	North	NM	Limestone	Open shrubland with *Pistacia lentiscus* and *Juniperus phoenicea*	8.98° W	37.03° N	7
Vela (PVE)	North	NM	Granite	*Pinus pinaster* plantation	7.29° W	40.43° N	9

Lineage and Type were defined on the basis of analyses published in Quintela-Sabarís *et al.* 2010. *Lineage*: cpSSR lineage ('North', 'South'); *Type*: M (metallicolous), NM (non-metallicolous). Geographic coordinates are given in decimal degrees. *N*: number of plants genotyped with AFLP in each population.

liferous soils (Pollard *et al.* 2002).

Due to the aforementioned duality of metalliferous areas, pseudometallophytes are interesting organisms since i) they constitute highly relevant models to study local adaptation in plants (Linhart and Grant 1996); and ii) they usually possess, along with metal tolerance, several traits (high adaptability to adverse soil conditions, high biomass production, good competitiveness…) that may make them useful for phytoremediation technologies (Poschenrieder *et al.* 2001).

The population genetics of different pseudometallophyte species has been investigated in order to identify evolutionary and genetic factors involved in tolerance (Westerbergh and Saura 1992, Vekemans and Lefèbvre 1997, Mengoni *et al.* 2001, Jiménez-Ambriz *et al.* 2007, Pauwels *et al.* 2008).

These studies mainly tried to identify whether metallicolous populations suffered a founder effect during the colonisation of metalliferous areas or to determine if metallicolous populations of a particular species share a common ancestry or whether they are the result of local colonization events. Their results show that metallicolous populations are usually the result of local evolution. However, regarding the occurrence of founder effect, a common trend across species was not found. Pauwels *et al.* (2005) proposed that

the colonisation of metal-polluted environments is associated with a genetic bottleneck in species with populational tolerance (e.g. *Silene paradoxa*, Mengoni *et al.* 2001), whereas in species with constitutive (or 'specieswide') tolerance (such as *Arabidopsis halleri*, Pauwels *et al.* 2005) the effect of a bottleneck may not be detected.

Among the molecular markers applied to non-model pseudometallophytes, those based on maternally-inherited chloroplast DNA (such as chloroplast microsatellites- cpSSR), are useful to infer the phylogeography of a species, making possible a better understanding of the effect of metal pollution on the genetic structure of its populations (Staton *et al.* 2001). However, chloroplast markers are mainly neutral and seldom related to metal tolerance.

In contrast, AFLP markers (Vos *et al.* 1995), which have predominantly a nuclear origin (Meudt and Clarke 2007) can be applied to any organism without previous knowledge of sequences. Thus, this kind of marker has allowed genome scans to be applied to non-model organisms (Bonin *et al.* 2007). The use of genome scans along with environmental data allows the detection of those markers potentially linked to adaptive loci (Holderegger *et al.* 2008) among many markers (such as AFLP). Thus, AFLP loci with potential ecological relevance have been identified in *Arabis alpina* (Poncet *et al.* 2010), or, interestingly, Meyer *et al.* (2009) have inferred loci putatively involved in tolerance to heavy metals in metallicolous (M) and non-metallicolous (NM) populations of the pseudometallophyte *Arabidopsis halleri*.

Cistus ladanifer L. (Cistaceae) is a pseudometallophyte shrub native to the Western Mediterranean region (from S of France to the N of Morocco and Algeria, Demoly and Montserrat 1993). In addition to its adaptability to disturbances occurring in Mediterranean areas, mainly fires (Pérez-García 1997) and water and light stress (Martín Bolaños and Guinea López 1949, Núñez-Olivera *et al.* 1996), this species has been described as interesting for phytostabilisation procedures and also for phytoextraction of Zn in soils with low to medium contents of this metal (Díez-Lázaro 2008).

In a previous paper we analysed 33 NM and M (serpentine and mines) *Cistus ladanifer* populations with cpSSR markers, from almost its entire distribution area (Quintela-Sabarís *et al.* 2010). We inferred that M populations evolved in parallel within two independent chloroplast lineages.

In this work, we re-analyse the same 33 populations with AFLP markers with two main aims: i) to assess and to compare the genetic structure and the patterns of colonisation of metalliferous areas by *C. ladanifer* obtained using AFLPs (nuclear markers, dispersed by pollen and seeds) and cpSSRs (maternally-inherited, and thus dispersed only through seeds (Guzmán and Vargas 2009)) analyses, and ii) to identify AFLP loci potentially associated to tolerance to metalliferous soils in this species.

We addressed the latter topic through the analysis of correlation be-

Table 6.2: Summary of amplification results and range of fragment sizes finally used in the analyses. Error rates were computed as indicated in Bonin *et al.* (2004). The correlation size/frequency is an indication of possible size homoplasy (Vekemans *et al.* 2002).

		Range of fragment sizes		
		50-500 bp	**100-500 bp**	**150-500 bp**
N of fragments	Combination A	64	57	50
	Combination B	80	73	60
	Total	**144**	**130**	**110**
Error rate	Combination A	0.074	0.069	0.064
	Combination B	0.054	0.055	0.048
	Total	**0.063**	**0.061**	**0.055**
Correlation Size/Frequency	Pearson index	-0.2653	-0.2168	-0.1292
	P	**0.001**	**0.013**	**0.178**

P: probability of obtaining a more extreme correlation value than that presented by chance alone.
Combination A: FAM-EcoRI- ACT/ MseI- CTG; ***Combination B***: NED-EcoRI- AGG/ MseI- CAG

tween the distribution of AFLP markers and total metal contents in soils and applying generalized estimating equations (GEE) to correct for phylogeographic autocorrelations of individuals within chloroplast lineages.

6.2 Material and Methods

6.2.1 Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire natural geographic range of this species were sampled. The subspecies growing in each site were identified on the basis of morphological traits. We included M populations from different geographic areas: ultramafic outcrops of Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), M populations growing on mine tailings and in the vicinity of highways from the Centre and South of the Iberian Peninsula and non-metallicolous (NM) populations (Table 6.1).

To collect the plant material a longitudinal transect was established for each population. Ten plants, separated by at least 5 m, were selected along each transect, and ripen fruits were collected from them. The seeds were sown and seedlings grown in the laboratory. One seedling per mother plant was selected for subsequent analyses. Young plants were frozen in liquid nitrogen and kept at -20ºC until DNA extraction.

In addition, in each site one (or two) soil samples were collected from 5 to 15 cm in depth. Each soil sample was air-dried and sieved through a 2 mm-mesh.

6.2.2 Soil analysis and variables

We initially considered nineteen soil variables for our statistical analyses: soil pH,

ratio of total Ca:Mg, ratio of extractable Ca:Mg, total content of As and Sb, and total and extractable contents of Co, Cr, Cu, Mn, Ni, Pb, and Zn (See Tables S 3.1 and S6.1 in Supplementary Material).

Total concentrations of Ca and the metalloid As were quantified in solid soil subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF), whereas other subsamples were digested with HNO_3 for the quantification of total Mg contents with Inductively Coupled Plasma -Optical Emission Spectrometry (ICP-OES) and for the quantification of total Sb with ICP-Mass Spectrometry (ICP-MS). Extractable Ca and Mg were quantified with Atomic Absorption Spectrometry in liquid soil extracts obtained following Quintela-Sabarís *et al.* (2010). Data of pH and total and extractable contents of the other metals were previously published (Quintela-Sabarís *et al.* 2010).

We performed a Principal Component Analysis on these variables in order to check the correlation among them. We finally retained only the most uncorrelated variables from the PCA: soil pH, Ca:Mg ratio and total soil contents of Mn, Ni, Pb, Sb and Zn. These seven variables were then used as explanatory soil variables in the identification of loci under directional selection.

6.2.3 DNA extraction

The DNA was extracted from 100 mg of frozen leaves using Dneasy® Plant Mini Kit (QIAGen), following the manufacturer's indications. In some cases an additional wash with 500 µl of absolute ethanol was needed in order to remove secondary compounds from the DNA extracts.

6.2.4 AFLP protocol and data scoring

To optimize the AFLP conditions and to select the primer-enzyme combinations to use, a preliminary study with 30 individuals from geographically separated populations and 64 combinations of selective primers was carried out using AFLP Plant Mapping Kit (Applied Biosystems). After the optimization procedure, we selected two combinations of selective primers that yielded reliable polymorphic banding patterns: **combination A** (EcoRI- ACT/MseI-CTG) and **combination B** (EcoRI- AGG/MseI- CAG). In addition, we introduced the following modifications in the manufacturer's protocol: (1) each restriction-ligation reaction was diluted with 11.0 µl of $TE_{0.1}$ buffer rather than 189.0 µl, (2) pre-selective amplification products were diluted with 40.0 µl $TE_{0.1}$ buffer instead of 190.0 µl, (3) each selective amplification was performed in a final volume of 12.5 µl instead of 25 µl.

Fluorescent labelled selective PCR products were separated using an ABI 3130 xl (Applied Biosystems) DNA

Table 6.3: Measures of intrapopulation genetic diversity. **PPL**: percentage of polymorphic loci. H_j: Nei's genetic diversity (and its standard deviation); **SMC**: Simple Matching Coefficient (and its standard deviation). In addition, we present data of genetic diversity computed on the basis of cpSSR in the same populations included in this study (Quintela-Sabarís *et al.* 2010).

Pop	Type	AFLP allele-based measures			AFLP band-based measure		cpSSR measures (Quintela-Sabarís et al. 2010)		
		PPL	H_j	S.D.(H_j)	1-SMC	S.D. (1-SMC)	H_E	$r_{(7)}$	D^2_{SH}
EAC	M	22.7	0.06	0.01	0.07	0.03	0.00	0.00	0.00
EAL	M	32.7	0.11	0.02	0.12	0.04	0.00	0.00	0.00
EBE	NM	36.4	0.11	0.02	0.12	0.05	0.64	2.33	0.76
ECA	NM	31.8	0.09	0.01	0.08	0.03	0.71	1.93	0.89
ECO	M	33.6	0.10	0.01	0.10	0.03	0.56	1.00	0.28
ECR	NM	30.9	0.08	0.01	0.08	0.03	0.36	0.93	0.18
EDE	M	34.5	0.11	0.02	0.10	0.04	0.64	1.70	0.46
EFS	NM	33.6	0.11	0.02	0.09	0.03	0.64	1.70	0.46
EGJ	NM	36.4	0.12	0.02	0.10	0.03	0.47	0.99	0.23
EGR	NM	31.8	0.08	0.01	0.08	0.03	0.00	0.00	0.00
EGU	NM	37.3	0.09	0.01	0.08	0.03	0.20	0.70	0.10
EMA	NM	30.0	0.08	0.01	0.06	0.02	0.62	1.87	1.78
EMF	NM	26.4	0.07	0.01	0.06	0.02	0.00	0.00	0.00
ERB	NM	30.0	0.10	0.01	0.07	0.02	0.53	1.00	0.27
ESB	M	38.2	0.11	0.02	0.10	0.06	0.00	0.00	0.00
ESP	M	27.3	0.08	0.01	0.07	0.03	0.42	1.56	0.50
ETO	M	26.4	0.07	0.01	0.04	0.03	0.20	0.70	0.10
EVC	NM	30.0	0.09	0.01	0.07	0.02	0.71	2.39	0.71
MBH	NM	31.8	0.10	0.01	0.07	0.04	0.20	0.70	0.10
MBT	NM	31.8	0.10	0.01	0.08	0.03	0.20	0.70	0.10
MEJ	NM	24.5	0.07	0.01	0.06	0.04	0.00	0.00	0.00
MKE	NM	25.5	0.08	0.01	0.06	0.03	0.38	1.40	0.22
MSI	M	22.7	0.07	0.01	0.05	0.02	0.53	1.00	0.27
MSII	M	30.0	0.09	0.01	0.08	0.04	0.70	2.16	0.58
MTA	NM	31.8	0.09	0.01	0.08	0.02	0.00	0.00	0.00
PAL	M	28.2	0.09	0.01	0.08	0.03	0.64	1.78	0.53
PBR	M	41.8	0.14	0.02	0.14	0.06	0.53	1.00	0.27
PBU	NM	40.0	0.13	0.02	0.13	0.06	0.39	0.97	0.19
PCF	NM	47.3	0.15	0.02	0.17	0.07	0.39	0.97	0.19
PMC	M	31.8	0.10	0.01	0.09	0.04	0.00	0.00	0.00
PSA	M	37.3	0.12	0.02	0.11	0.05	0.00	0.00	0.00
PSV	NM	42.7	0.15	0.02	0.15	0.05	0.20	0.70	0.10
PVE	NM	42.7	0.13	0.02	0.11	0.03	0.00	0.00	0.00
Mean (±S.D.)		32.7 ± 6.0	0.098 ± 0.024		0.090 ± 0.030		0.32 ± 0.27	0.92 ± 0.77	0.28 ± 0.37

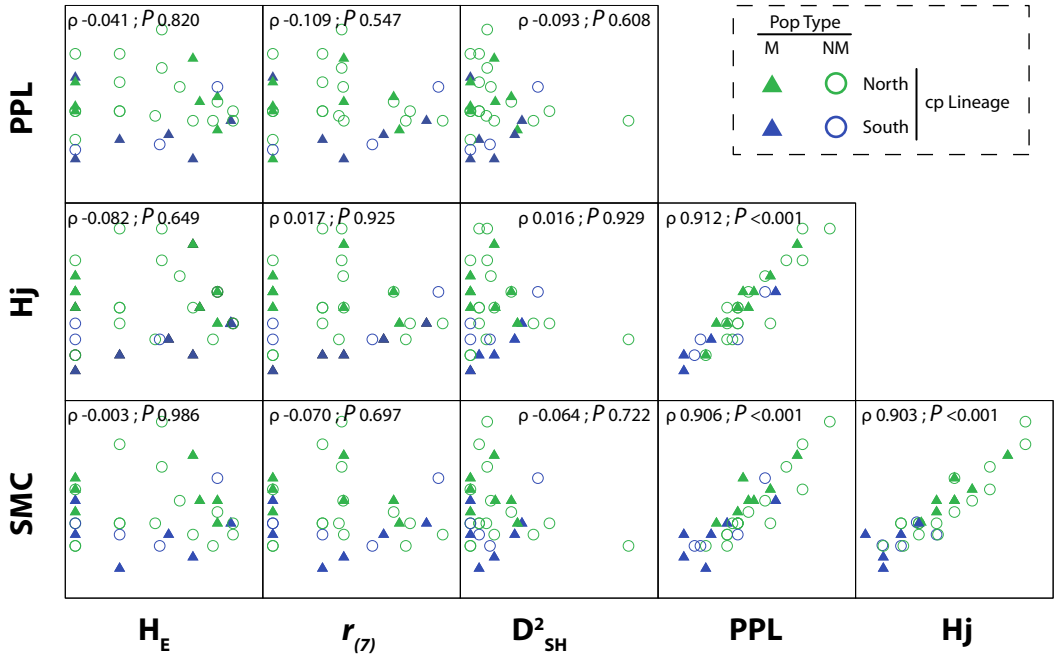


Figure 6.1: Relationships among different AFLP-based estimates of intrapopulation genetic diversity and others estimates of genetic diversity based on cpSSR markers (Quintela-Sabarís *et al.* 2010). Different symbols indicate population type (M and NM) and chloroplast lineage (North or South). Based on AFLP: **PPL**, % of polymorphic loci; **Hj**, Nei's gene diversity; **SMC**, 1-(simple matching coefficient). Based on cpSSR: $\mathbf{H_E}$, haplotypic diversity (Nei 1987); $\boldsymbol{r_{(7)}}$, haplotype richness after rarefaction to a population size of 7 plants (El Mousadik and Petit 1996); $\boldsymbol{D^2_{SH}}$ measure (Vendramin *et al.* 1998). The value of Spearman's $\boldsymbol{\rho}$ ('rho') correlation index and ***P*** (probability of obtaining a more extreme ρ value than that presented by chance alone) are presented in each graph.

analyser. The ABI files with electropherograms were visualized and subsequently scored using the open source program Genographer v 2.0 (Benham 2001, modified by Travis Banks and available at http://sourceforge.net/projects/genographer/). Genographer converts the electropherograms into a gel-like image which is easier to score by visual screening. Afterwards, we applied a correction, which eliminates many of the slight deviations that exist from lane to lane, and resulted effective in fixing compressions or expansions along the length to the lane.

The AFLP markers have 2 alleles, so were scored as band presence (1) or band absence (0). Those plants showing weak or very awkward profiles were removed from the analysis. The quality of PCR amplifications and bin scoring was assessed as following: 20 plants (6.5% of plants analysed) were re-extracted and amplified independently. An error rate was computed as the sum of errors/the total number of comparisons (Bonin *et al.* 2004). Those bins with an error rate equal or higher than 0.2 were removed from the analysis.

Table 6.4: Results of the non-parametrical Mann-Whitney tests on the intrapopulation genetic diversity data. Comparisons between M (metallicolous) and NM (non-metallicolous) populations were made for the whole set of populations and separately for each chloroplast lineage. For each estimator of genetic diversity (**PPL**: % of polymorphic loci. H_j: Nei's gene diversity. **SMC**: 1-Simple Matching Coefficient), we present: ***U***, Mann-Whitney statistic. m_{NM} and m_M refer to the median value of the estimator for the groups of Non-Metallicolous (NM) and for Metallicolous (M) populations, respectively. n_{NM} and n_M refer to the number of populations of NM and M populations within each group. ***P***: probability of obtaining a more extreme *U* value than that presented by chance alone.

Div Estimator	Data set	*U*	m_{NM}	n_{NM}	m_M	n_M	*P* (2-tailed)
PPL	Cp lineage 'North'	48.0	32.70	14	33.60	7	0.940
	Cp lineage 'South'	12.0	31.80	6	26.85	6	0.332
	All populations	108.5	31.80	20	31.80	13	0.426
H_j	Cp lineage 'North'	41.0	0.10	14	0.11	7	0.547
	Cp lineage 'South'	12.0	0.09	6	0.08	6	0.328
	All populations	120.5	0.10	20	0.10	13	0.724
SMC	Cp lineage 'North'	31.0	0.08	14	0.10	7	0.175
	Cp lineage 'South'	14.0	0.08	6	0.07	6	0.515
	All populations	122.0	0.09	20	0.08	13	0.766

Depending on the selective primer combinations, markers were named A or B, followed by a number indicating its fragment size (in bp). Initially, all fragments between 50 and 500 bp were considered, but, as posterior analyses revealed size homoplasy in the lowest bp ranges (as previously found by Vekemans *et al.* 2002; see following section), only the fragments from 150 bp to 500 bp sizes were considered.

6.2.5 Data analysis: within population diversity and genetic structure of populations

The genetic diversity within population estimates were computed based on two different approaches: the allele frequency-based metrics (% of polymorphic loci, **PPL**; and the Nei's gene diversity per locus, H_j, after Lynch and Milligan (1994)) and the band-based metric (Simple Matching Coefficient, **SMC**).

Allele frequency-based metrics were computed using AFLP-SURV 1.0 software (Vekemans 2002). To estimate allele-frequencies, we used a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), and assumed a Hardy-Weinberg genotypic proportions ($F_{IS} = 0$). This is a reasonable assumption, since *Cistus ladanifer* is an obligatory-outcrossing species, with a gametophytic mechanism of incompatibility (Talavera *et al.* 1993), and in allogamous species allele frequencies usually do not violate the Hardy-Weinberg Equilibrium (Meudt and Clarke 2007).

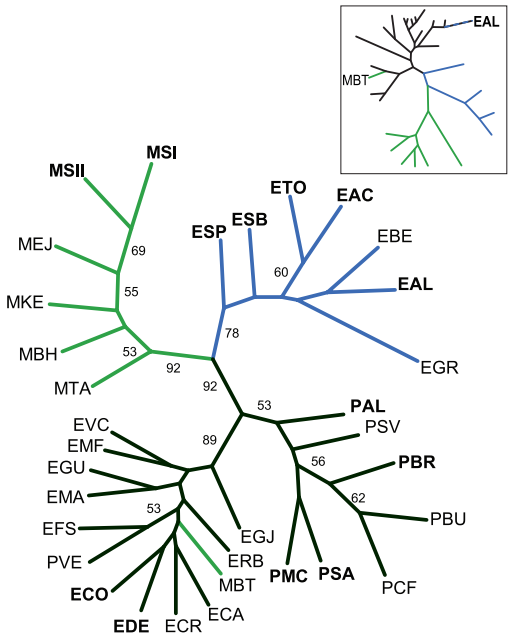


Figure 6.2: Unrooted consensus dendrogram based on 1000 bootstrapped trees. Population codes are the same as table 6.1. Nei's D distance between populations (after Lynch and Milligan 1994) was used. Numbers indicate the percentage of support for each branch of the dendrogram. Only bootstrap values higher than 50 are reported. A scheme of the consensus dendrogram obtained previously with cpSSR on the same populations (Quintela-Sabarís *et al.* 2010) is presented in the upper right corner of the figure. Green lines indicate the position of populations from the North of Morocco, whereas blue lines indicate populations from the Betic area (South and South-east of the Iberian Peninsula). M populations are indicated by **bold type**.

We computed the Pearson correlation coefficient between fragment size and fragment frequencies on the overall sample with the same software (Vekemans *et al.* 2002). After preliminary analyses with different size ranges, non significant values of Pearson coefficient were obtained using fragments in the range 150-500 bp (Table 6.2), so we selected this range to perform the statistical analyses.

The Simple Matching Coefficient (SMC) was computed within each population using the SIMQUAL module from NTSYS-pc software (v. 2.11L; Rohlf, 2002). The SMC, which takes into account both shared 1's (presence of a band) and shared 0's (absence of a band), was proposed as the best metric for comparisons of phenotypic similarity with dominant markers within a single diploid species (Kosman and Leonard, 2005). SMC is a measure of similarity, therefore we computed the dissimilarity for each population as: (1-SMC).

Differences in the levels of intrapopulation diversity between M and NM populations were assessed using the nonparametrical Mann-Whitney test, which is equivalent to Student's t-test, but without the assumption of the normality of data and a better robustness against outliers. Separate analyses were performed on the whole set of 33 populations and separately for each chloroplast lineage, using PPL, Hj and SMC data.

The estimates of intrapopulation genetic diversity obtained with AFLP markers were compared with those obtained previously with cpSSR (Quintela-Sabarís *et al.* 2010) using the Spearman correlation index, which does not request data normality. The Mann-Whitney tests and the Spearman correlation indices were performed with the SPSS package (v. 15, SPSS Inc., Chicago, IL, USA).

A pairwise matrix of genetic distances between populations was com-

puted based on Nei's genetic distance, D (after Lynch and Milligan 1994), using the AFLP-SURV. The genetic distances were validated by a bootstrap procedure with 1000 replications. Each bootstrapped matrix was used to compute a Neighbour-Joining (NJ, Saitou and Nei 1987) dendrogram with the program NEIGHBOR. The program CONSENSE allowed us to construct a consensus dendrogram on the basis of 1000 NJ dendrograms. NEIGHBOR and CONSENSE belong to the package PHYLIP (v. 3.6, Felsenstein 2004).

We computed the correlation between pairwise D matrix (obtained with AFLP) with (a) the matrix of genetic distance obtained with cpSSR markers (based on Cavalli-Sforza and Edwards distances, Quintela-Sabarís *et al.* 2010), and with (b) a matrix of pairwise geographic distances (expressed as natural logarithm of Km, Ln(km)) using the Mantel matrix-correspondence test (Mantel 1967), which is included in the module MXCOMP from NTSYS-pc (v. 2.11L; Rohlf, 2002). We set 10000 permutations to validate the results.

In order to infer population genetic structure, Bayesian analysis using a spatial clustering model implemented in BAPS software version 5.4 was performed (Corander *et al.* 2008). The spatial clustering of groups model was run using each population, with known coordinates, as the unit to be clustered. We initially fixed *k* (the number of clusters) from 2 to 33. Afterwards, we selected the value of *k* that had the minimum log marginal likelihood and repeated the analysis 100 times to obtain the optimal population structure. The use of spatial information increases the power to correctly detect the underlying population structure (Bonin *et al.* 2007).

The results of the mixture analysis were then used to perform an admixture analysis, following the protocol by Corander and Marttinen (2006). We used the following settings: (1) minimal size of clusters. five individuals; (2) 200 iterations to estimate the admixture coefficients for the individuals; (3) 300 simulated reference individuals from each population, and (4) 20 iterations to estimate the admixture coefficients for the reference individuals. According to Corander and Marttinen (2006) BAPS performs equally well or even better than the widely used program STRUCTURE (Pritchard *et al.* 2000) with a 400-fold speed advantage.

The population genetic structure was further explored using a locus-by locus Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992), implemented with the Arlequin 3.5 software (Excoffier *et al.* 2005). This test allowed for estimation of variance components between individuals within populations, between populations within groups and among groups. The groups were defined either on the basis of soil analyses or on the basis of BAPS-clustering. Variance components and Φ statistics were estimated for each locus and then combined to produce synthetic estimators of Φ statistics. The significance values were computed by a permutation test from 20000 permuted matrices.

Finally, we computed the pollen-to-seed migration ratio($r = m_p/m_s$) follow-

ing Petit *et al.* (2005). We estimated the genetic subdivision at nuclear markers (Φ_{STb}) using Arlequin 3.5 software (Excoffier *et al.* 2005), whereas the subdivision at maternally inherited markers (Φ_{STm}) was obtained from a previous study (Quintela-Sabarís *et al.* 2010).

6.2.6 Data analysis: detection of ecologically relevant loci using GEE

We used generalized estimating equations (GEE) in order to detect alleles that were correlated to soil variables. GEE are an extension of generalized linear models (Carl and Kuhn 2007), which may consider autocorrelation between samples by including an additional variance component directly into the independent data model's estimating equation to accommodate correlated data. This method allows us to consider that neighbouring individuals within chloroplast lineages are genetically more similar than individuals belonging to different chloroplast lineages which, as we inferred in a previous work (Quintela-Sabarís *et al.* 2010), may be isolated from each other from the Last Glacial Maximum at least. As we dealt with binary data, we used a logit-link and binomial error distribution to correlate allele occurrence for each AFLP locus per sampling location to quadratic polynomials of environmental variables. To consider the variety of response curve shapes other than a linear response (Legendre and Legendre 1998) we used quadratic polynomials. In order to select the best GEE models, we used the quasi-likelihood information criterion (QIC) adapted by Pan (2001). The best model is that with the lowest QIC. All combinations of variables were investigated for each locus. Finally, we tested 128 GEE (from the null model to the full model with seven variables in second-order polynomials) on our data set. All GEE models were calculated using the R package *geepack* (Yan and Fine 2004), and an implementation developed by Poncet *et al.* (2010) for the QIC calculation in R (R Development Core Team 2007). For each selected model, we also tested each regression coefficient using the Wald test. To avoid a high rate of false positives due to multiple tests, significance levels were calculated for each regional data set by minimizing the false discovery rate (FDR; Storey 2002). *P*-value rejection thresholds were adjusted so as to have less than one false positive locus per data set and were estimated using the R package *qvalue*.

6.3 Results

A total of 109 and 112 different fragments were amplified with primer combinations A and B, respectively. However, after the filtering process (error rates and size ranges) 110 markers were finally considered (50 obtained with combination A and 60 with combination B). The total error rate using both combinations was 0.055 (Table 6.2).

6.3.1 Genetic diversity and differentiation

The values of genetic diversity within populations are presented in Table 6.3. The Mann-Whitney tests did not reveal significant differences in diversity between M

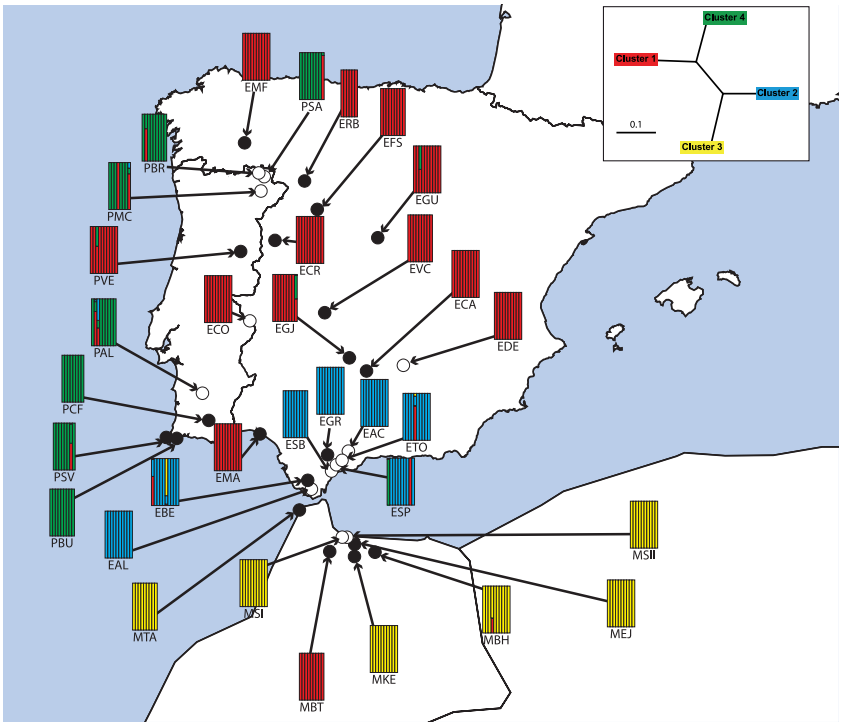


Figure 6.3: Localities for the 33 sampled populations of *Cistus ladanifer* (for details of each population see Table 6.1). Black circles indicate NM populations whereas white circles indicate M populations. The graphs next to each population indicate the proportional assignment of individuals from each population to the four different clusters as detected in an admixture analysis of AFLP data conducted with the program Bayesian Analysis of Population Structure (BAPS). Every vertical coloured bar corresponds to an individual. A vertical bar is split into several colours when there is evidence for admixture. The BAPS-defined clusters are: cluster 1 (red), cluster 2 (blue), cluster 3 (yellow) and cluster 4 (green). Only significant admixtures (significance level 5%) are showed. A NJ dendrogram showing the relationships among each of those clusters is presented on the upper right corner. NJ dendrogram is based on Kullback-Leibler distances among clusters.

and NM populations, neither when considering the whole set of populations nor each chloroplast lineage separately (Table 6.4).

We found high and significant linear correlations among the three diversity estimates computed on AFLP data. Interestingly, the estimates of genetic diversity based on AFLP and cpSSR were not correlated (Figure 6.1).

The matrix of Nei's D genetic distances among populations is available in Supplementary Material S 6.2. As previously obtained with chloroplast markers, the consensus dendrogram revealed a clustering more related to phylogeography than to soil type (Fig. 6.2). A group of populations of *C. ladanifer* subsp. *africanus* from the North of Morocco is defined (marked in green colour in Fig.

Table 6.5: Summary of analysis of molecular variance (AMOVA) of *Cistus ladanifer* (a) considering the whole data set, (b) be tween population types (M vs. NM), (c) between BAPS-defined groups (*K* = 4) and (d to g) separate analyses between population types for each of the four groups defined by SAMOVA analysis. *SS* = sum of squared deviation, ***P*** = level of probability of obtaining a more extreme component estimate by chance alone. n.s. = not significant (5% level).

Analysis	Source of variation	SS	Variance components	% Total Variance	*P*
(a) Whole data set (Φ_{ST}= 0.35)	Among populations	836.74	2.62	35.47	< 0.0001
	Within populations	1126.61	4.77	64.53	
	Total	1963.35	7.39		
(b) M vs NM	Between population types (Φ_{CT} = 0.01)	40.87	0.11	1.52	0.02
	Among pops. within population types	795.87	2.57	34.44	< 0.0001
	Within populations	1126.61	4.77	64.04	< 0.0001
	Total	1963.35	7.45		
(c) BAPS mixture results	Among BAPS-defined clusters (Φ_{CT} = 0.31)	515.69	2.50	31.04	< 0.0001
	Among pops. within clusters	321.05	0.77	9.62	< 0.0001
	Within populations	1126.61	4.77	59.33	< 0.0001
	Total	1963.35	8.04		
(d) Cluster 1	Between population types (Φ_{CT} = 0.01)	12.68	0.06	1.06	n.s.
	Among pops. within population types	117.23	0.74	13.72	< 0.0001
	Within populations	435.09	4.57	85.22	< 0.0001
	Total	565.00	5.37		
(e) Cluster 2	Between population types (Φ_{CT} = -0.03)	9.97	-0.14	-2.84	n.s.
	Among pops. within population types	66.39	1.01	20.12	< 0.0001
	Within populations	229.43	4.16	82.71	< 0.0001
	Total	305.79	5.03		
(f) Cluster 3	Between population types (Φ_{CT} = 0.002)	13.03	0.01	0.16	n.s.
	Among pops. within population types	52.45	1.11	22.63	< 0.0001
	Within populations	159.39	3.78	77.21	< 0.0001
	Total	224.87	4.90		
(g) Cluster 4	Between population types (Φ_{CT} = 0.01)	9.77	0.07	1.00	n.s.
	Among pops. within population types	39.52	0.15	2.19	0.04
	Within populations	302.70	6.78	96.81	0.01
	Total	351.99	7.01		

6.2). In addition, another cluster groups all the populations from South and SE of the Iberian Peninsula (Betic Area, marked in blue in Fig. 6.2). Populations from the rest of Iberian Peninsula are arranged in two other clusters. Population MBT from the North of Morocco, which belongs to *C. ladanifer* subsp. *ladanifer* is located next to populations of the same subspecies from Iberian Peninsula. Each of the four defined clusters includes both M and NM populations.

The congruency between AFLP and chloroplast markers is also supported by the fact that a Mantel test (1967) revealed that genetic distances among populations based on AFLP or on cpSSR were positively correlated (the value of correlation factor r was 0.5688; *P* 0.0001). However, the population EAL showed differences between AFLP and cpSSR: on the basis of AFLP it is placed in a cluster with other populations from the Betic area, but if we consider cpSSR this population is more linked to populations from SW to the North of Iberian Peninsula (Fig. 6.2). Mantel test revealed a low but significant correlation between matrices of genetic and geographic distances between populations (r = 0.3923; $P < 0.0001$).

Bayesian mixture analysis inferred four clusters of populations (k = 4; Log(marginal likelihood) of optimal partition: -6 099.8331) which fit the four clusters identified in NJ consensus dendrogram (Fig. 6.3). The degree of admixture among clusters is low, and it occurs mainly in populations from the Iberian Peninsula. It is remarkable the fact that no individual plant from population MBT (*C. ladanifer* subsp. *ladanifer* from N of Morocco) showed indications of admixture with the north African cluster 3 (marked in yellow). In contrast population EBE (*C. ladanifer* subsp. *africanus* from S of Iberian Peninsula) presents an indication of admixture with the cluster 3. A with the consensus dendrogram, each of the four defined clusters includes both M and NM populations (Fig. 6.3).

The AMOVA analysis inferred that most of the molecular variance occurs at the intra population level (64.53%; Table 6.5a), and a Φ_{STb} value of 0.35 ($P < 0.0001$). AMOVA also revealed a significant partitioning of 31.04% of molecular variance among the four clusters defined by BAPS ($\Phi_{CT} = 0.31$; $P < 0.0001$; Table 6.5c). In contrast, the comparisons between population types at the global level (Table 6.5b) revealed that less than 2% of molecular variance occurs between M and NM populations. The differentiation between population types became non-significant when the AMOVA analyses where performed within each BAPS cluster (Table 6.5d to 6.5g).

6.3.2 Detection of relevant loci using GEE

GEE were used to correlate allele frequencies to soil variables correcting for chloroplast lineages. The threshold for significance minimizing the false discovery rate was estimated as 0.0031. Sixteen loci (14.5% of total loci) were significantly correlated to one or two soil variables (Fig. 6.4), giving a total of 21 significant

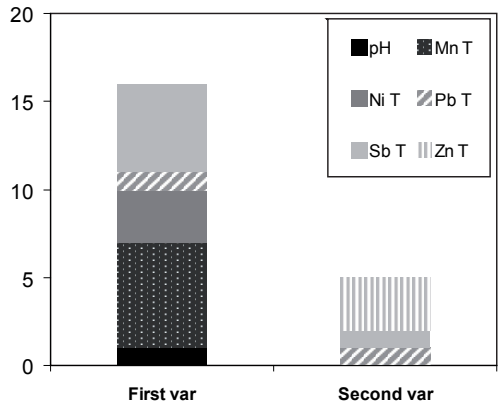


Figure 6.4: Number of AFLP loci significantly correlated with soil variables in the generalized estimating equations (GEE) applying a correlation for chloroplast lineage. Sixteen bands (14.5% of obtained loci) were correlated with one variable, 5 of which were also correlated with a second variable. Different fill patterns represent pH and Total soil contents of Mn, Ni, Pb, Sb and Zn.

relationships (Supplementary Material S 6.3). MnT and SbT were the soil variables with most influence, since each of them represented 28.6% of detected significant correlations. NiT and ZnT accounted for 14% each. No locus was significantly related to Ca:Mg ratio.

In order to prune possible false positives, we further explored these 21 relationships by i) performing separate Mann-Whitney U tests to detect significant differences in soil variables between the group of plants where a certain band was absent or present, and ii) observing the geographic distribution of the presences/absences of each locus.

The Mann-Whitney U tests showed significant differences for only 8 loci (5 of them related to MnT), whereas in the case of the loci A492 and B346 they were marginally significant ($0.05 < P < 0.10$) (Table 6.6).

The distribution maps of band presences in relation to soil variables show that several bands (e.g. A183, B293, B320, B485 in relation to PbT,…) appear mainly in populations with low metal contents, and thus, those bands may not really be related to metals. However, two bands (B401 and B485) are present mainly in populations with higher soil contents of manganese. The map of B401 presences is presented in Fig. 6.5, whereas the maps for the other loci are in Supplementary Material S 6.4.

6.4 Discussion

6.4.1 Comparison of AFLP and cpSSR inferences of genetic diversity and differentiation

In this work we present a species-wide analysis of *C. ladanifer* using a genome scan of AFLP markers.

We have obtained an estimation of genetic differentiation ($\Phi_{ST} = 0.35$) which is much higher than data for other species (above the 3rd quartile in data from 77 species, Petit *et al.* 2005) and is congruent with the trends inferred by Nybom (2004) from species with similar life history traits (perennial, outcrosser, barochorous and from early-mid successional status).

Moreover, we estimated a quite low pollen/seed migration ratio, which indicates that the dispersal of seeds have accounted for a large component of the historical gene flow between populations (above the 45%). In fact, Metcalfe and

Table 6.6: Summary of results of the Mann-Whitney test on potentially environmental related loci detected by GEE. Only those loci with significant (or near significant) results are presented. ***U***: Mann-Whitney statistic. m_a and m_p refer to the median value of the related variable for the group of plants where the AFLP marker was absent or present, respectively. n_a and n_p refer to the number of plants where each AFLP marker was absent or present, respectively. ***P***: probability of obtaining a more extreme *U* value than that presented by chance alone.

Locus	*Related Variable*	*U*	m_a	n_a	m_p	n_p	*P* (2-tailed)
A183	SbT	5302.0	0.014	189	0.012	81	< 0.001
A189	pH	1101.5	6.30	250	6.99	50	< 0.001
A339	NiT	2626.5	45.99	240	27.86	30	0.016
A492	NiT	1417.5	45.99	255	18.69	15	0.092
B293	MnT	1273.0	532.7	252	241.9	16	0.013
B320	MnT	2677.0	532.7	238	424.8	30	0.026
B346	SbT	3688.0	0.012	229	0.014	39	0.082
B391	MnT	742.5	478.4	258	2805.0	10	0.001
B401	MnT	2614.5	454.9	233	1577.5	35	0.001
B485	MnT	1065.0	478.4	252	2191.3	16	0.002
B485	PbT	997.0	17.75	252	11.59	16	0.001

Kunin (2006) reported that pollination success in isolated *C. ladanifer* plants dropped to 0 when the distance to the nearest neighbour was around 3.5 m.

This near equal contribution of seed and pollen flow may explain the fact that, in spite of the different properties (such as mutation rate, ploidy level, homoplasy, inheritance) of AFLPs and cpSSRs, the matrices of inter-population genetic distances based on each type of markers were significantly correlated.

As a consequence of matrix correlations, similar NJ and Bayesian clusterings were obtained with both markers. The only discrepancy between markers was observed in the population EAL, located in a contact zone between different glacial lineages (Quintela-Sabarís *et al.* 2010).

We may consider this case as an example of the aforementioned properties of maternally-inherited cpDNA (e.g. Comes and Kadereit 1998), which conserve the history of colonisation, whereas in the case of nuclear markers this history is blurred by recombination or by pollen flow from nearby populations.

Similarities in population clustering also implies that AFLPs tell the same story as cpSSRs about the origin of the metallicolous (M) populations of *C. ladanifer*: they are the result of multiple and independent colonisation events that, in addition, did not imply any genetic differentiation related to soil type, as indicated by the AMOVA results within each BAPS-

defined cluster. Instead, and given the significance of the Mantel test on geographic and genetic distances, the genetic differentiation may be explained better as a result of historical processes (i.e. location and isolation of glacial refugia) together with isolation-by-distance. In this case, the significant difference between M and NM populations revealed by species-wide AMOVA can be interpreted as a spurious result produced by a "covariation" of phylogeography and soil type: each type of population has a different relative weight within each cluster inferred by BAPS. Hence, and following Staton *et al.* (2001), we have to underline the need to understand the phylogeography of a species as a prerequisite to correctly interpreting the effect of stress factors (heavy metals, in our case) on its genetic structure.

Similarly, studies which applied AFLPs to other pseudometallophytes also revealed the lack of an effect of soil type on genetic differentiation (e.g. *Cerastium velutinum*- Gustafson *et al.* 2003; *Onosma echiodes*- Mengoni *et al.* 2006; *Armeria maritima*- Baumbach and Hellwig 2007).

From a taxonomic point of view, and like previous cpSSR results (Quintela-Sabarís *et al.* 2010), the populations from S and SE Andalusia (Betic area) actually form a cluster separated from the other populations, nevertheless they are identified as belonging to subsp. *ladanifer* on the basis of leaf morphology. In contrast, the populations of the subsp. *sulcatus* (formerly *Cistus palhinhae*), despite showing several morphological differences, do not form a single cluster and are instead grouped together with populations of subsp. *ladanifer*.

Regarding subsp. *sulcatus*, our results are in contrast with those obtained by Carlier *et al.* (2008), who, analysing populations from the Algarve region (S of Portugal) with AFLPs and ISSRs, inferred a genetic differentiation between subsp. *ladanifer* and subsp. *sulcatus*. Our differences may be explained by the fact that Carlier *et al.* (2008) analysed DNA samples bulked for each population and also because the ISSR markers had an important effect, since they showed some alleles exclusive to one of the two subspecies considered. However, they obtained a low differentiation between subspecies (Dice index 0.98; Carlier *et al.* 2008).

Overall, these results point to the need for a taxonomic revision of the intraspecific taxa in *C. ladanifer*, especially in order to recognize the value and unique genetic features of Betic populations of this species.

As indicated by the AMOVA analyses, most of the genetic variation revealed by AFLPs occurs at the intrapopulation level, as would be expected for nuclear markers in an obligate-outcrossing species such as *C. ladanifer* (Loveless and Hamrick 1984, Nybom 2004). The assessment of genetic diversity within populations may suffer bias due to the dominant nature of AFLP markers. In order to overcome this possible bias, we have used SMC, the best metric for dominant markers in diploid species (Kosman and Leonard 2005), and we complemented SMC with two allele frequency-based diversity

estimators (PPL and Hj), computed using a robust Bayesian approach (Zhivotovsky 1999). Thus, we have obtained reliable estimators of genetic diversity, giving the similarities and significant correlations among the three indices.

As we previously inferred with cpSSRs, AFLPs detected no differences in genetic diversity related to soil type, a trend that seems to be common in the analyses of pseudometallophytes with nuclear-DNA markers (Mengoni *et al.* 2000, Mengoni *et al.* 2006, Baumbach and Hellwig 2007; but also some exceptions, e.g. Deng *et al.* 2007). This fact points to a lack of selective constraints on metallicolous populations of *C. ladanifer* and, thus, it may be an additional support on our consideration of tolerance to heavy metals as a constitutive trait in this species (Quintela-Sabarís *et al.* 2010).

However, we have to keep in mind that we are working with more than one hundred (putatively) independent and neutral markers, so possible selective effects in few markers may be diluted by the other neutral ones.

Ribeiro *et al.* (2002) found a correlation of genetic diversity estimations obtained with either AFLPs or cpSSRs in *Pinus pinaster*. These authors attributed this congruence to the fact that in this species gene flow through pollen (cpDNA is paternally-inherited in *P. pinaster*) is more important than marker-specific factors. In the case of *C. ladanifer*, the maternally-inherited cpDNA is not influenced by pollen flow, so the lack of correlation among the estimates of within-population genetic diversity obtained with AFLP markers and those obtained with cpSSRs, may be caused by the previously mentioned different properties of each kind of markers and the different effect of processes (such as genetic drift, recombination…) on them.

6.4.2 Detection of relevant loci using GEE

We have applied generalized estimating equations (GEE) to our data in order to reveal loci potentially related to soil variables (pH, Ca:Mg ratio, but especially heavy metal contents in soils). Contrary to other methods for detecting loci under selection, which usually rely on prior assumptions about population structure, migration and mutation rates, and also involve the estimation of F_{ST} from allele frequencies (see Bonin *et al.* 2007, and references therein), GEE directly correlate the allele distribution of each marker independently with environmental variation to produce 'allele distribution models'. Thus, these models are also individual-based and hence insensitive to biases caused by low sample size (Holderegger *et al.* 2008), an issue especially interesting for our study, with a medium number of plants per population. Although we are analysing the relationships between soil conditions and AFLP, other two main factors may include "noise" on our analyses: phylogeography and climatic variation. We have corrected the possible influence of phylogeography by introducing a correcting factor based on the previous information about chloroplast lineages (Quintela-Sabarís *et al.* 2010). Regarding the climate, PCA analyses on

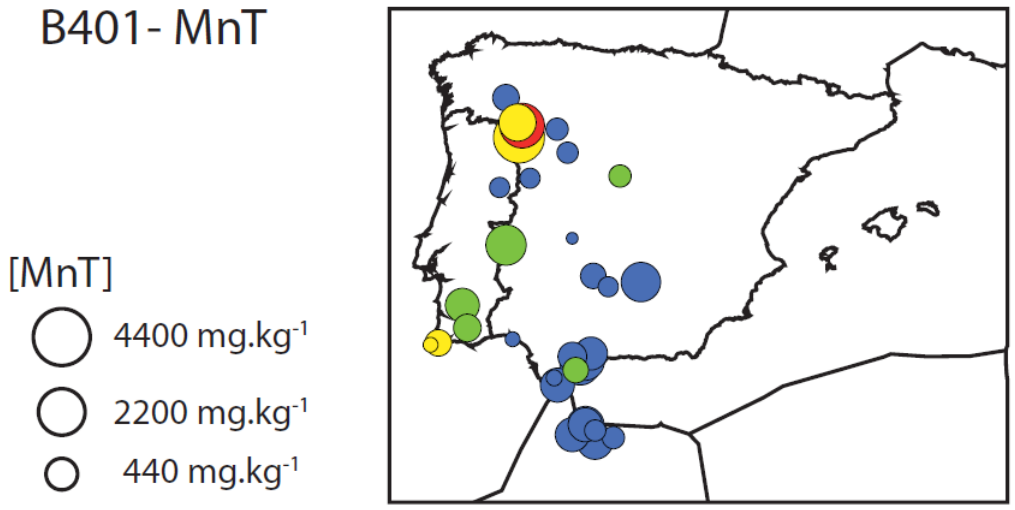


Figure 6.5: Geographic distribution of loci B401, potentially related to MnT. The geographic distribution of the band presence overlays the spatial variation of MnT. The diameter of each circle is proportional to the value of MnT in each population. The colour of the circle represent for each population the frequency (***f***) of plants in which the AFLP-fragment is present: blue (***f*** = 0); green (0 < ***f*** ≤ 0.50); yellow (0.50 < ***f*** ≤ 0.75) and red (0.75 < ***f***).

19 bioclimatic variables from each population revealed no clines or patterns on our populations (data not shown), so we may conclude that the allele distribution models mainly reflect the influence of soil variables.

Regarding soil variables, it is remarkable that we did not detected any loci related to Ca:Mg ratio, which points to a lack of selective effect of this soil variable on *Cistus ladanifer*. High levels of Mg in relation to Ca are considered one of the main stress factor to plants growing on serpentine soils (e.g. Brady *et al.* 2005). This unexpected result is congruent with previous reports signalling the low Ca requirements of *C. ladanifer* as a competitive trait allowing this plant growing in serpentine soils (Alados *et al.* 1999), and the high Mg requirements of *C. ladanifer* plants growing on serpentine soils from N of Morocco(Ater *et al.* 2000).

The opposite situation to Ca:Mg ratio is MnT, the total content of manganese in soils, which was shown to be the most influential of the soil variables. Its effect is even higher than other variables with a similar variation range (up to several thousand $mg \cdot kg^{-1}$ in soil; e.g. NiT and PbT), and may be related to the higher mobility in soils of Mn compared to Ni or Pb (Friedland 1990).

Manganese is a micronutrient essential to plant metabolism. It has functions in photosynthesis and also a protective role against oxidative stress (Epstein and Bloom 2005), but it may be toxic at high concentrations, provoking chlorosis

or necrosis. Thus, plants have to regulate the uptake of this element and its translocation in plant body in order to fulfil plant requirements but also in order to keep Mn levels below the threshold of safety for this metal (Wenzel *et al.* 2004).

Cistus ladanifer has been described in the literature as a Mn accumulator (Alvarenga *et al.* 2004, de la Fuente *et al.* 2010). Moreover, in a broadscale study, we have estimated that populations of *C. ladanifer* from the chloroplast lineage 'North' have higher levels of Mn in leaves and higher Mn leaf:soil ratios than populations from lineage 'South' (unpublished results), so this species must have developed mechanisms to regulate the uptake and translocation and thus, the tolerance to this metal, especially in some areas where the soil content of Mn is especially high (such as serpentine areas from NE Portugal or mine tailings from the C of Iberian Peninsula).

Among the loci whose distribution was significantly related to MnT, we have strong confidence in B401 being linked to genes involved in the tolerance to increasing levels of Mn. We support this assertion on the basis that i) the 'present' allele is more frequent in populations with high MnT values, and ii) these populations are distributed in a wide geographic area, thus avoiding local effects. Interestingly, most of the populations where B401 is present belong to cpLineage 'North', with higher Mn accumulation (see previous paragraph).

However, we are conscious of the fact that this does not prove of the adaptive relevance of marker B401, so, in accordance with Holderegger *et al.* (2008), subsequent analyses, which may include the isolation, sequentiation and identification of this band or the development of reciprocal transplant experiments, are needed in order to prove the adaptive or selective advantage of this band in relation to Mn.

In summary, we have obtained results that have allowed us to conclude that AFLPs provide similar inferences to those obtained by cpSSRs on the phylogeography and the colonisation of metalliferous areas by the pseudometallophyte *C. ladanifer* (multiple colonisation, independent origin, lack of genetic differentiation related to soil type), although there are some differences, related to the different properties of each kind of marker. In addition, we have proved the potentialities of GEE analysis, since it allowed us to estimate which soil variables explained more allele distributions in *C. ladanifer*, as well as to detect a band with a possible role in tolerance to high Mn concentrations in soils.

Acknowledgements

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Final Synthesis and Conclusions

Previous page: General view of a shrubland community growing on soils developed from metabasic rocks, near Vila Verde (Trás-Os-Montes, NE Portugal). This area was recently burned and *Cistus ladanifer* subsp. *ladanifer* has now become the dominant plant. (Photo: PS Kidd)

Final Synthesis and Conclusions

As was presented in previous chapters, we have conducted a series of studies on phylogeography, population genetics and ecophysiology of the Mediterranean pseudometallophytic species *Cistus ladanifer* (rockrose).

On the basis of a broad scale analysis of chloroplast microsatellites (cpSSRs), we have come to the conclusion that genetic diversity in this widespread species is geographically structured in two (or three, depending on the statistical methodology employed) main lineages or clusters.

These clusters are, in congruence with pollen data from the bibliography, the result of postglacial recolonisation of this species from isolated refugia located in N of Morocco, SW Iberian Peninsula and SE Spain.

Moreover, we have inferred that metallicolous populations of this species are the result of multiple and independent processes of colonisation. Interestingly, the colonisation of metalliferous areas has not left genetic imprints related to soil type (in the form of genetic bottlenecks or founder effects). This fact constitutes the first piece of evidence to support the theory that tolerance to heavy metals is a species-wide trait in rockrose.

There are a great number of local and regional studies on the accumulation of heavy metals by *C. ladanifer*. The application of phylogeographic knowledge allowed us to perform a study at a species-scale level, separating lineage and soil effects.

We have not observed different metal contents between metallicolous (M) and non-metallicolous (NM) populations of this species for a series of metals, except Ni. A plausible explanation may be the occurrence of mechanisms for restricted metal accumulation in M populations.

Notwithstanding the previous assertion, we have found different patterns of accumulation of heavy metals among M populations from different chloroplast lineages. This phenomenon, already observed in other pseudometallophytes, both reflects and supports the independent history of M populations, which have evolved in parallel within lineages that have been isolated since the Last Glacial Maximum.

However, and in spite the fact taht the species *C. ladanifer* clearly rejects the accumulation of the metals Co, Cr and Pb, we have noticed considerable differences among particular populations in response to other metals. This means that any phytostabilization procedure using this plantshould be preceded by a survey that allows the characterization of its local ecotypes in relation to heavy metals, in order

to prevent a rockrose-mediated transfer of metals into the ecosystems' food chain.

Our research carried out on tolerance to Co, Ni and Zn, in hydroponic cultures, revealed that each metal affected plants in a different way. Metal effects are congruent with the patterns of metals accumulation/exclusion we observed from field samples. Thus, for future analysis of metal tolerance in plants it would be useful to know the strategy of response to heavy metals in a given species in order to determine the best parameter to be measured.

Under the conditions of our hydroponics experiment we observed no differences in most response variables (growth, biomass, chlorophyll fluorescence) among M and NM populations. This fact may be interpreted as a second piece of evidence for the tolerance to metals as a constitutive (species-wide) trait in *C. ladanifer*.

Again, different chloroplast lineages also implied different patterns or mechanisms of response to metals. However, given the observed effects of metal treatments on response variables, we suggest that a possible preadaptation to nutrient shortage and water stress, instead of true metal tolerance, may have facilitated the colonisation of metalliferous soils by *C. ladanifer*.

Although the properties of AFLP markers differ with respect to cpSSRs (diploid, biparentally-inherited genome vs. haploid, maternally-inherited), they provided similar inferences on the phylogeography of the species. Moreover, AFLPs revealed no influence of soil type on the genetic diversity and differentiation of this species.

The GEE procedure was shown to be a useful statistical tool that allowed us to consider molecular data and soil information together. According to evidence provided by different authors on the nutrient requirements of *C. ladanifer*, we perceived no effect of Ca:Mg ratio (one of the most important factors in serpentine stress) on the distribution of AFLP markers. In contrast, we found that Mn content in soils has the strongest effect on allele distribution among the variables we analysed. In fact, we report the detection of a band with a possible role in tolerance to high Mn soil content, although future research in this area is needed.

Some final remarks to improve the present knowledge of *Cistus ladanifer*

Finally, we would like to suggest some future research lines in order to look for answers to questions that are still unresolved:

- ***Root symbioses and tolerance***: there is increasing evidence on the important role of soil microorganisms in the tolerance of plants to metals. In our case, this area deserves more attention, given the fact that *C. ladanifer* is a pseudometallophyte with several known ectomycorrhizal fungi and where some Plant Growth Promoting Rhizobacteria (PGPR) have been de-

scribed.

- ***Development of landscape genetic analysis using AFLP markers***: The Alentejo area (in fact, all the Iberian pyrite belt) provides an interesting scenario to study the evolution and adaptation to seriously stressing soil conditions at a landscape scale. As we have shown in this Ph.D. Thesis, allele distribution models on AFLP data provide potentially useful tools to advance in this area.

- ***Integrate phylogeography into chemoecology of the species***: leaf exudates provide beneficial effects to *C. ladanifer*. It has been proved that a seasonal and a regional variation in these exudates exists. It would be interesting to investigate whether isolation among chloroplast lineages also resulted in differences in exudates.

Complete Bibliography

This section comprises all the bibliographic references cited in the text (all chapters).

Previous page: *Cistus ladanifer* subsp. *ladanifer* var. *albiflorus* plants growing next to the Ni-hyperaccumulator *Alyssum serpyllifolium* (small-yellow flowers) on ultramafic outcrops in Samil (Trás-Os-Montes, NE Portugal). (Photo: PS Kidd)

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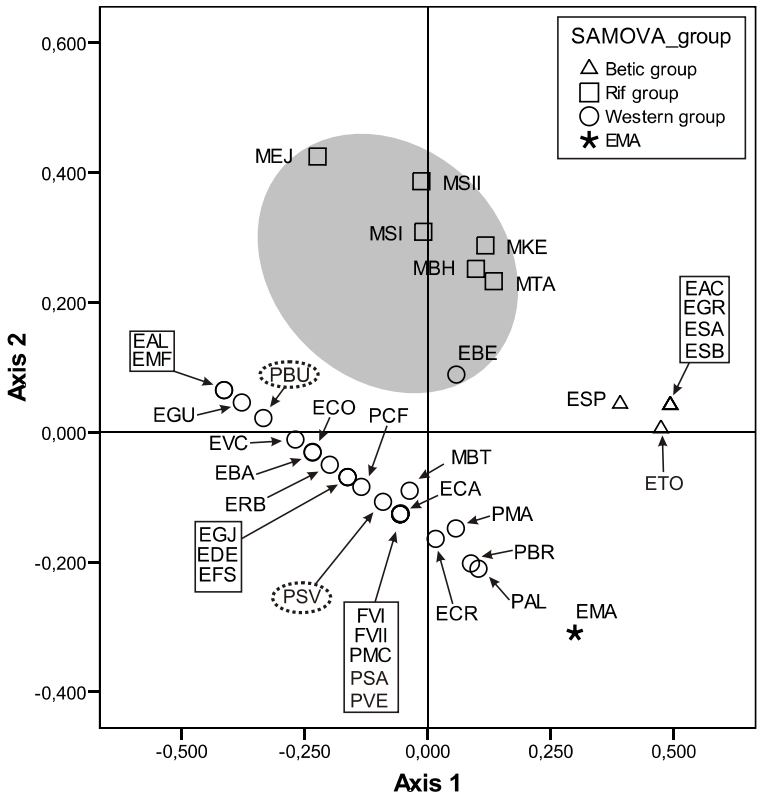
Supplementary material

This section includes tables and figures which complement the information presented in chapters 2-6.
Tables and figures are numbered using the format: **Supplementary material S *x.y***; where ***x*** is the chapter number and ***y*** is the order of the table/figure in the chapter ***x***.

Previous page: Drop of *labdanum* on a young stem of *Cistus ladanifer*, near Grazalema (Cádiz province, S of Spain). *Labdanum* provides protection against herbivores and also against excessive insolation. (Photo: C. Quintela-Sabarís)

Supplementary material S 2.1: Principal Coordinate Analysis (PCoA) results. Populations were plotted along the two first PCoA axes. For population codes, see Table 2.1. Different symbols are used for each SAMOVA-defined group. Grey shading indicates populations of *C. ladanifer* subsp. *africanus*. Population codes encircled by a dotted line indicate populations of *C. ladanifer* subsp. *sulcatus*. In some cases, population codes are connected to their plot by an arrow. Population codes inside squares indicate populations occupying exactly the same position.



Supplementary material S 2.2: Bibliographical sources of occurrences of *Cistus ladanifer* pollen. These sources were used to construct the map presented in Figure 2.4. First we present a table with a summary of each paper. Then, on next page, the complete bibliographical references are presented.

Reference	Site	Type of deposit	Long	Lat	Datation
Fletcher *et al.* (2007)	CM5 borehole (Guadiana Valley, Algarve, Portugal)	Sediment core	-7.45	37.27	First peak around 12,000 BP and another from 4,040 to 2,830 BP
Franco-Múgica *et al.* (2001)	Espinosa de Cerrato (Palencia, Spain)	River Franco marsh	-3.94	41.96	First peak around 8,000 BP and then new peaks from 2,700 BP to present times
Franco-Múgica *et al.* (2005)	El Carrizal lake (Segovia, Spain)	Sediment core from lake deposit	-4.15	41.32	Around 2,500 BP
López-Sáez *et al.* (2007)	Los Barruecos (Cáceres, Spain)	Archaeological site	-6.50	39.42	Around 6,000 BP
López-Sáez *et al.* (2009)	Portlligat Bay (Girona, Spain)	Sediment core from Posidonia oceanica living bed	3.29	42.29	Around 1,300 BP
Nocete *et al.* (2008)	Valencina de la Concepción (Guadalquivir Valley, Seville, Spain)	Archaelogical site, smelting quarter	-6.07	37.41	4,150 to 4,045 BP
Pons and Reille (1988)	Padul (Granada, Spain)	Radiocarbon dated peat core	-3.67	37.00	90,000 cal BP
Stevenson (2000)	Ojos del Tremedal (Montes Universales, Teruel, Spain)	Radiocarbon dated peat core	-2.05	40.54	1,830 to 440 cal BP
Van der Knaap and Van Leeuwen (1995)	Charco da Candieira (Serra da Estrela, Portugal)	Sediment core from lake deposit	-7.58	40.34	2,685 BP
Van der Knaap and Van Leeuwen (1997)	Lagoa Comprida 2 (Serra da Estrela, Portugal)	Sediment core from lake deposit	-7.64	41.32	Around 10,000 BP
Van der Schriek *et al.* (2007)	Muge River (Near Santarém, Portugal)	Sediment core in river bank	-8.66	39.1	From 5,800 to 5,200 cal BP

Longitude and *Latitude* are presented in decimal degrees. *BP*: years before present. *cal BP*: calibrated years before present

Supplementary material S 2.2: (continued)

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- López Sáez JA, López García P, López-Merino L, Cerrillo Cuenca E, González Cordero A, Prada Gallardo A (2007) Origen Prehistórico de la Dehesa en Extremadura: una Perspectiva Paleoambiental. Revista de Estudios Extremeños 63:493-510
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- Van der Knaap WO, Van Leeuwen JFN (1997) Late Glacial and early Holocene vegetation succession, vegetation zonation, and climatic change in the Serra de Estrela, Portugal. Review of Palaeobotany and Palynology 97:239-285.
- Van der Schriek T, Passmore DG, Stevenson AC, Rolão J (2007) The palaeogeography of Mesolithic settlement-subsistence and shell midden formation in the Muge valley, Lower Tagus Basin, Portugal. The Holocene 17:369-385.

Supplementary material S 3.1: Total and extractable contents of metals in soils of the populations analysed in chapter 3. Population Codes and assigned Groups are the same as in table 3.1. Refer to Fig. 3.1 to see the ordination of the populations obtained by a Principal Component Analysis of metal contents. <*d.l.* indicates values under detection limit
4

Pop Code	Group	Total metal contents (µg.g^{-1})							Extractable metal contents (µg.g^{-1})						
		Co	Cr	Cu	Mn	Ni	Pb	Zn	Co	Cr	Cu	Mn	Ni	Pb	Zn
EAC	M(u)	89	2054	31	994	1893	33	62	68	1	7	670	183	12	<d.l.
EAL	M(h)	17	127	24	1119	33	13	70	14	0.55	11	930	11.5	9	9
EBE	NM	3	53	3	94	8	17	15	<d.l.	<d.l.	<d.l.	138	<d.l.	<d.l.	11
ECA	NM	6	48	4	216	58	33	26	<d.l.	<d.l.	<d.l.	152	<d.l.	<d.l.	5
ECO	M(h)	22	87	103	2127	57	43	85	12	<d.l.	68	2500	12	16	21
ECR	NM	3	33	5	210	0	28	33	<d.l.	<d.l.	<d.l.	430	<d.l.	15	14
EDE	M(h)	36	90	28	1824	37	174	123	28	<d.l.	9	2000	6	169	60
EFS	NM	5	48	3	242	14	18	20	<d.l.	<d.l.	<d.l.	161	<d.l.	<d.l.	<d.l.
EGJ	NM	24	120	29	455	46	49	123	<d.l.	<d.l.	<d.l.	264	<d.l.	<d.l.	<d.l.
EGR	NM	8	146	17	658	65	8	36	8	<d.l.	<d.l.	1030	10	<d.l.	5
EGU	NM	5	12	7	304	0	50	61	<d.l.	<d.l.	27	272	<d.l.	<d.l.	12
EMA	NM	3	79	32	80	19	14	15	<d.l.	<d.l.	18	24	<d.l.	6	5
EMF	NM	9	104	42	533	47	44	83	<d.l.	<d.l.	20	79	<d.l.	6	7,5
ERB	NM	7	70	7	273	3	65	28	8	<d.l.	<d.l.	400	<d.l.	91	17
ESB	M(u)	58	1518	23	525	715	17	81	12	0.6	<d.l.	133	67.5	10	8
ESP	M(u)	140	3204	24	1957	2914	27	84	62	0.75	<d.l.	740	250	5	9
ETO	M(u)	201	3803	21	1672	2963	32	58	181	0.95	<d.l.	1800	580	18	<d.l.
EVC	NM	3	34	11	34	3	16	16	<d.l.	<d.l.	<d.l.	61	<d.l.	<d.l.	<d.l.
MBH	NM	10	29	7	279	15	10	18	6	<d.l.	<d.l.	400	<d.l.	5	<d.l.
MBT	NM	21	178	27	1091	49	16	93	<d.l.	<d.l.	<d.l.	292	<d.l.	<d.l.	<d.l.
MEJ	NM	7	81	13	230	16	11	28	5	<d.l.	<d.l.	309	<d.l.	8	12
MKE	NM	22	122	40	1395	61	21	115	5	<d.l.	<d.l.	560	<d.l.	<d.l.	6
MSI	M(u)	88	2262	23	1201	1871	11	66	55	0.6	<d.l.	820	237	<d.l.	6
MSII	M(u)	123	2762	42	1598	2575	16	75	91	0.65	5	1070	350	<d.l.	<d.l.
MTA	NM	1	75	9	0	8	9	9	<d.l.	<d.l.	<d.l.	2	<d.l.	<d.l.	<d.l.
PAL	M(h)	17	75	269	1830	44	2168	892	40	<d.l.	75	2700	14	590	430
PBR	M(u)	69	944	56	1578	1151	4	76	5	<d.l.	<d.l.	140	8	<d.l.	<d.l.
PBU	NM	12	49	7	478	12	14	30	8	<d.l.	<d.l.	700	8	<d.l.	<d.l.
PCF	NM	21	79	24	602	51	16	55	5	<d.l.	<d.l.	650	5	<d.l.	<d.l.
PMC	M(u)	220	9248	47	4362	4573	18	170	89	<d.l.	5	1400	451	<d.l.	8
PSA	M(u)	150	6191	155	2805	1543	9	121	48	0.38	18	635	175.5	<d.l.	<d.l.
PSV	NM	2	30	5	69	11	5	8	<d.l.	<d.l.	<d.l.	75	<d.l.	<d.l.	5
PVE	NM	2	16	7	209	0	25	68	<d.l.	<d.l.	<d.l.	33	<d.l.	<d.l.	<d.l.

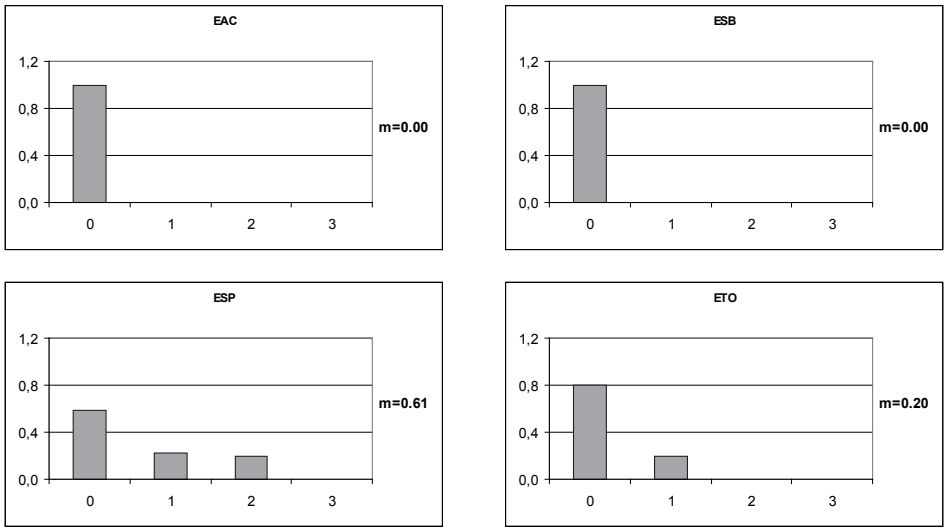
Supplementary material S 3.2: Definition of cpSSR haplotypes (chlorotypes) detected in *Cistus ladanifer*. Haplotype codes are the same as in Figure 3.2.

Allele Size (bp)		***Haplotype Code***	***N***
ccmp2	*ccmp3*		
136	113	**H1**	6
136	114	**H2**	20
136	115	**H3**	109
136	116	**H4**	72
136	117	**H5**	1
137	114	**H6**	46
137	115	**H7**	46
137	116	**H8**	21
138	115	**H9**	2
138	116	**H10**	1

N: number of plants displaying the haplotype

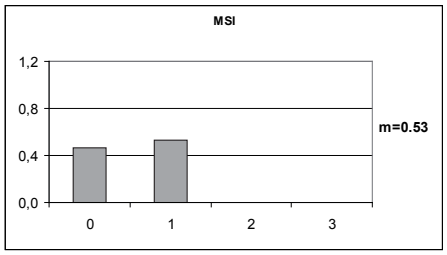
Supplementary material S 3.3: Distribution of pairwise chloroplast simple sequence repeats (cpSSR) length differences among plants. A graph is presented for each population (for population codes see table 3.1). Populations were arranged according to their chloroplast lineage ('North' or 'South') and their soil type (Metallicolous or Non-metallicolous). Ordinates represent the frequency; abscissa, the length difference. In addition, the mean difference (***m***) is indicated for each histogram.

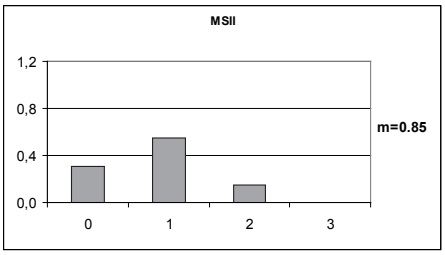
'South' Lineage, Metallicolous Populations



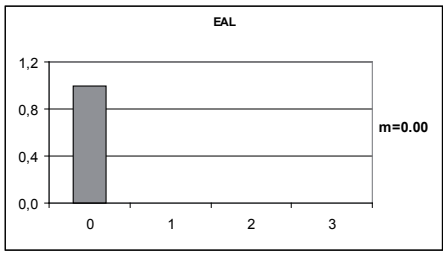
Supplementary material S 3.3: (continued)

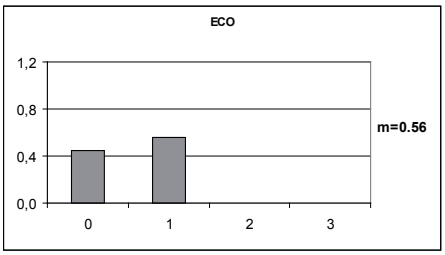
'South' Lineage, Metallicolous Populations

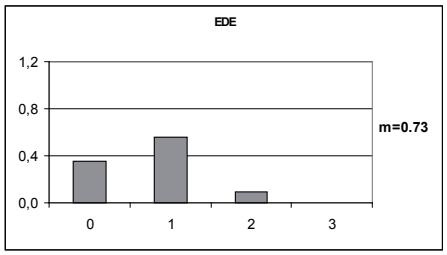


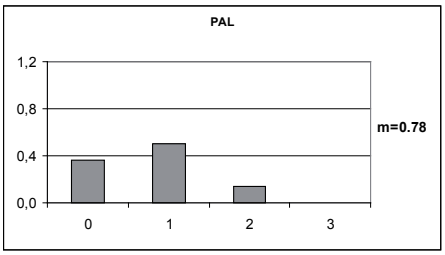


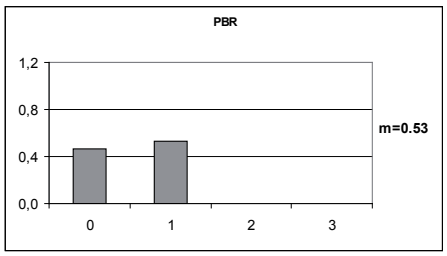
'North' Lineage, Metallicolous Populations

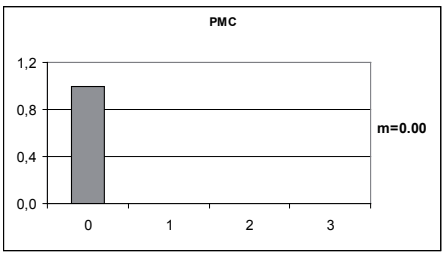


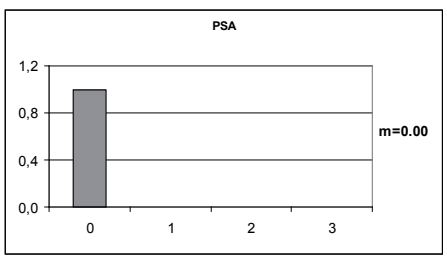




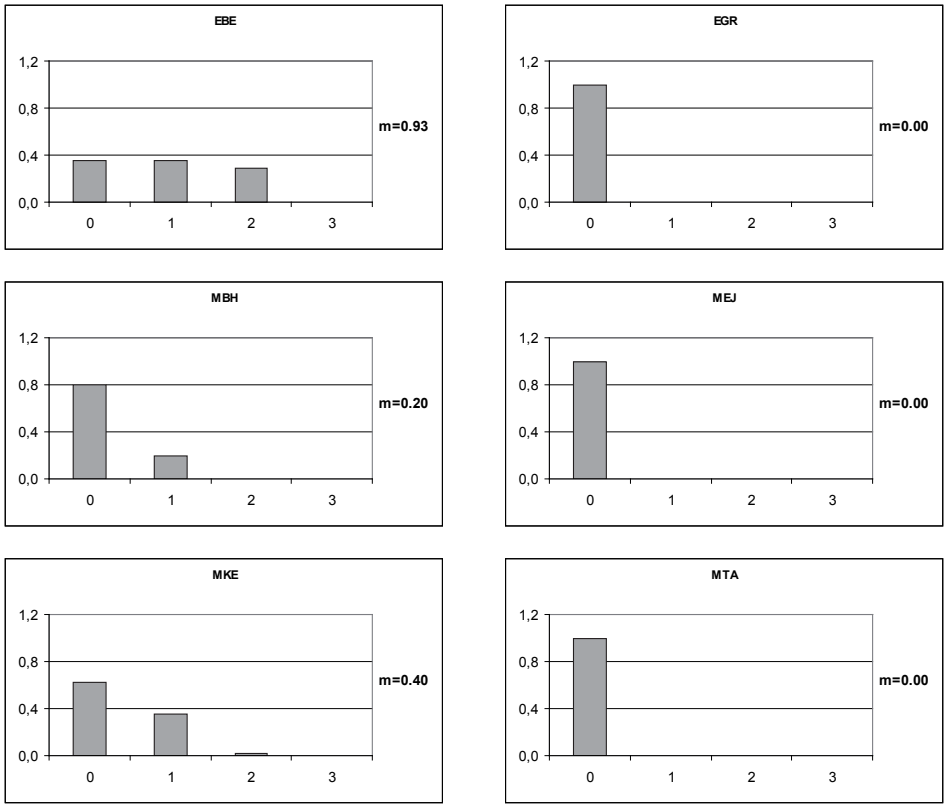




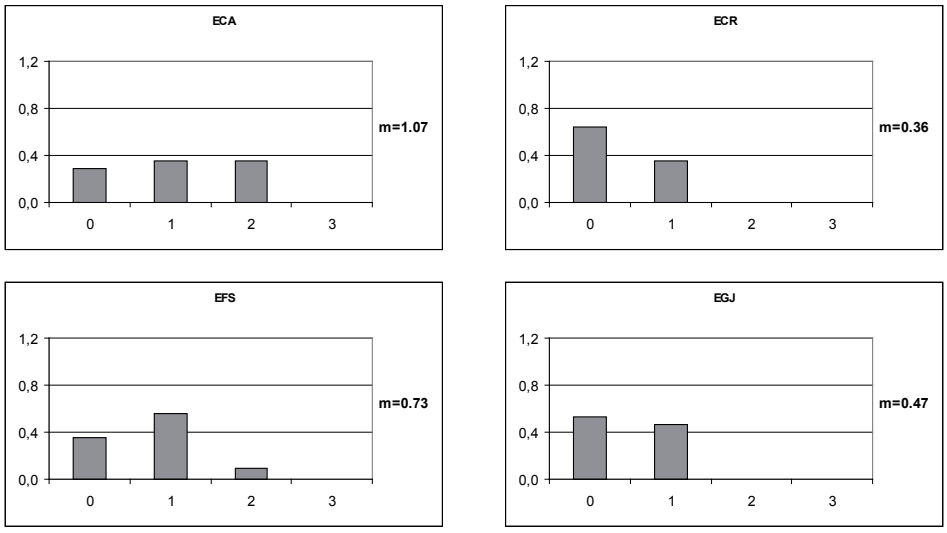




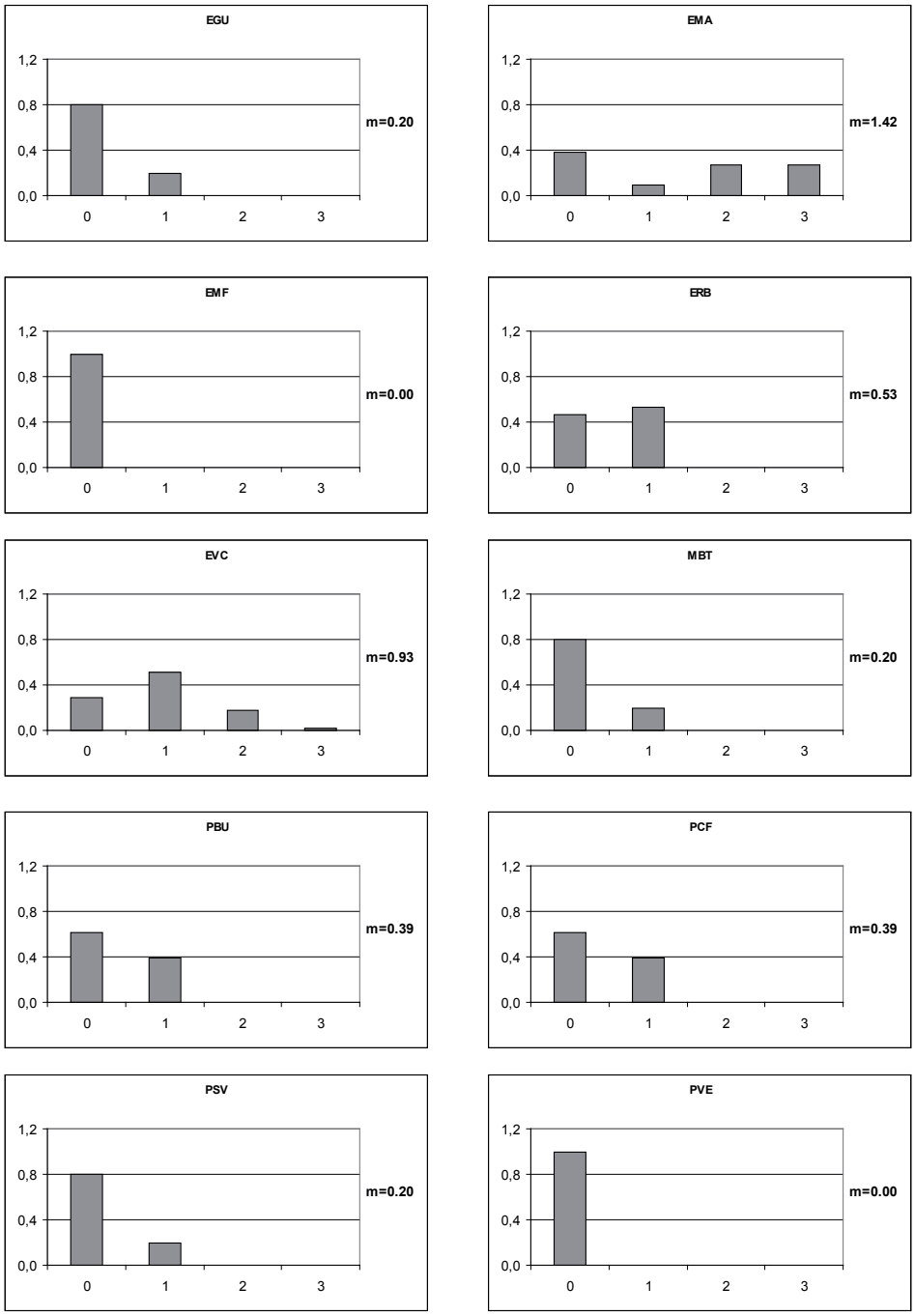
Supplementary material S 3.3: (continued)
'South' Lineage, Non-metallicolous populations



'North' Lineage, Non-metallicolous Populations



Supplementary material S 3.3: (continued)
'North' Lineage, Non-metallicolous Populations



Supplementary material S 5.1: Total and extractable contents of Co, Ni and Zn in soils of the populations included in the experiments of tolerance in hydroponic cultures. Pop Codes, and assigned Group and Lineages are the same as in table 5.1. Refer to chapter 3 in order to see the justification of Groups and Lineages.

Pop Code	Group	Lineage	Total metal contents (µg.g^{-1})			Extractable metal contents (µg.g^{-1})		
			Co	Ni	Zn	Co	Ni	Zn
EAC	M	South	89	1893	62	68	183	<d.l.
EBE	NM	South	3	8	15	<d.l.	<d.l.	11
ECA	NM	North	6	58	26	<d.l.	<d.l.	5
ECO	M	North	22	57	85	12	12	21
ECR	NM	North	3	0	33	<d.l.	<d.l.	14
EGJ	NM	North	24	46	123	<d.l.	<d.l.	<d.l.
EMA	NM	North	3	19	15	<d.l.	<d.l.	5
EMF	NM	North	9	47	83	<d.l.	<d.l.	7,5
ERB	NM	North	7	3	28	8	<d.l.	17
ESB	M	South	58	715	81	12	67,5	8
ESP	M	South	140	2914	84	62	250	9
ETO	M	South	201	2963	58	181	580	<d.l.
MBT	NM	North	21	49	93	<d.l.	<d.l.	<d.l.
MKE	NM	South	22	61	115	5	<d.l.	6
MSI	M	South	88	1871	66	55	237	6
MSII	M	South	123	2575	75	91	350	<d.l.
PAL	M	North	17	44	892	40	14	430
PBR	M	North	69	1151	76	5	8	<d.l.
PBU	NM	North	12	12	30	8	8	<d.l.
PCF	NM	North	21	51	55	5	5	<d.l.
PMA	NM	North	5	9	10	<d.l.	<d.l.	<d.l.
PMC	M	North	220	4573	170	89	451	8
PSA	M	North	150	1543	121	48	175,5	<d.l.
PSV	NM	North	2	11	8	<d.l.	<d.l.	5
PVE	NM	North	2	0	68	<d.l.	<d.l.	<d.l.

Extractable contents refer to Ammonium Acetate/EDTA/Acetic Acid, pH 4.65 ***<d.l.*** indicate values under detection limit

Supplementary material S 6.1: Summary of soil variables used for Principal Component Analysis. Data presented are pH, total and extractable Ca:Mg ratios, and total contents of metals As and Sb The total and extractable contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn are presented in Supplementary material S 3.1.

Pop Code	pH	Ca:Mg ratio		Metal contents (μg·g^{-1})	
		Total	Extractable	As	Sb
EAC	6.77	0.05	0.43	4.81	0.02
EAL	7.93	4.34	39.3	5.24	0.01
EBE	4.85	2.29	5.51	3.57	0.01
ECA	5.82	0.7	8.23	9.57	0.01
ECO	7.26	1.92	19.53	54.27	4.51
ECR	6.62	1.29	5.5	8.43	0.03
EDE	6.5	2.24	10.78	27.05	0.03
EFS	6.53	1.18	3.75	7.83	0.02
EGJ	5.8	0.09	2.82	26.31	0.01
EGR	6.92	2.3	20.61	9.68	0.01
EGU	6.31	1.67	6.85	1.51	0.01
EMA	6.01	5.91	11	11.31	0.11
EMF	5.41	2.23	9.59	30.71	0.06
ERB	5.41	2.25	12.57	6.78	0.03
ESB	5.98	0.19	0.89	7.87	0.01
ESP	5.06	0.04	1.86	1.21	0.01
ETO	6.99	0.02	0.79	3.93	0.01
EVC	4.96	1.48	6.55	9.55	0.07
MBH	6.58	0.98	12.22	3.51	0.01
MBT	5.18	0.17	1.86	4.4	0.01
MEJ	6.05	2.54	8.18	2.56	0.01
MKE	6.3	0.82	10.04	6.98	0.01
MSI	7.1	0.1	1.37	3.82	0.01
MSII	7.17	0.12	0.71	4.59	0.01
MTA	5.16	0.48	1.01	9.78	0.01
PAL	4.22	1.23	8.74	752.45	1.10
PBR	6.02	0.23	1.91	3.36	0.01
PBU	6.51	0.93	9.14	14.49	0.01
PCF	5.13	0.1	4.23	11.25	0.01
PMC	6.6	0.19	0.45	5.52	0.02
PSA	6.47	0.82	0.73	1.62	0.01
PSV	7.31	12.63	45.18	5.11	0.04
PVE	5.11	0.84	4.96	6.54	0.03

Supplementary material S 6.2: Matrix of population pairwise genetic distances. Below diagonal: Nei's D (alter Lynch and Milligan 1994) computed on AFLP data. Above diagonal: Cavalli-Sforza and Edwards (1967) distance computed on cpSSR data. cpSSR data were taken from chapter 3.

	EAC	EAL	EBE	ECA	ECO	ECR	EDE	EFS	EGJ	EGR	EGU	EMA	EMF	ERB	ESB	ESP
EAC		0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0	0.9	0.9	0.9	0.9	0	0.309
EAL	0.021		0.744	0.546	0.487	0.9	0.546	0.546	0.605	0.9	0.204	0.669	0	0.546	0.9	0.9
EBE	0.017	0.008		0.569	0.611	0.61	0.558	0.558	0.606	0.9	0.673	0.731	0.744	0.606	0.9	0.693
ECA	0.047	0.042	0.033		0.438	0.507	0.261	0.261	0.476	0.9	0.458	0.647	0.546	0.453	0.9	0.831
ECO	0.053	0.044	0.037	0.000		0.546	0.207	0.207	0.131	0.9	0.293	0.546	0.487	0.064	0.9	0.787
ECR	0.053	0.045	0.037	0.000	0.002		0.428	0.428	0.452	0.9	0.762	0.697	0.9	0.499	0.9	0.754
EDE	0.044	0.036	0.030	0.000	0.000	0.001		0	0.224	0.9	0.378	0.57	0.546	0.206	0.9	0.787
EFS	0.059	0.052	0.043	0.013	0.010	0.011	0.008		0.224	0.9	0.378	0.57	0.546	0.206	0.9	0.787
EGJ	0.040	0.035	0.023	0.003	0.007	0.006	0.003	0.008		0.9	0.418	0.556	0.605	0.067	0.9	0.765
EGR	0.013	0.010	0.004	0.033	0.043	0.036	0.035	0.046	0.022		0.9	0.9	0.9	0.9	0	0.309
EGU	0.060	0.054	0.041	0.012	0.015	0.014	0.012	0.019	0.001	0.040		0.593	0.204	0.355	0.9	0.852
EMA	0.059	0.054	0.041	0.003	0.006	0.002	0.005	0.013	0.002	0.039	0.002		0.669	0.548	0.9	0.831
EMF	0.052	0.047	0.035	0.006	0.010	0.006	0.010	0.017	0.000	0.031	0.006	0.001		0.546	0.9	0.9
ERB	0.057	0.051	0.039	0.000	0.002	0.000	0.003	0.010	0.001	0.038	0.003	0.000	0.000		0.9	0.775
ESB	0.019	0.018	0.008	0.034	0.038	0.040	0.032	0.044	0.024	0.003	0.044	0.043	0.034	0.040		0.309
ESP	0.011	0.018	0.006	0.029	0.038	0.032	0.031	0.040	0.016	0.000	0.033	0.032	0.025	0.031	0.005	
ETO	0.002	0.016	0.009	0.030	0.038	0.034	0.030	0.042	0.021	0.001	0.039	0.037	0.029	0.035	0.006	0.000
EVC	0.053	0.044	0.033	0.010	0.009	0.009	0.010	0.015	0.001	0.033	0.004	0.007	0.000	0.002	0.033	0.027
MBH	0.045	0.041	0.024	0.049	0.054	0.053	0.045	0.061	0.032	0.033	0.051	0.056	0.049	0.053	0.031	0.026
MBT	0.045	0.044	0.030	0.002	0.006	0.007	0.002	0.017	0.004	0.033	0.009	0.004	0.011	0.002	0.034	0.027
MEJ	0.047	0.052	0.030	0.053	0.062	0.057	0.051	0.066	0.038	0.036	0.059	0.058	0.053	0.058	0.037	0.028
MKE	0.040	0.044	0.020	0.044	0.051	0.048	0.044	0.054	0.028	0.024	0.047	0.049	0.041	0.047	0.024	0.018
MSI	0.052	0.059	0.036	0.059	0.069	0.063	0.059	0.071	0.043	0.040	0.061	0.064	0.057	0.063	0.044	0.035
MSII	0.045	0.049	0.029	0.053	0.063	0.053	0.052	0.066	0.037	0.032	0.057	0.057	0.050	0.056	0.037	0.029
MTA	0.059	0.065	0.044	0.063	0.070	0.068	0.058	0.078	0.049	0.047	0.069	0.068	0.061	0.067	0.042	0.043
PAL	0.034	0.040	0.022	0.024	0.028	0.028	0.025	0.035	0.008	0.019	0.018	0.024	0.017	0.022	0.017	0.010
PBR	0.045	0.049	0.031	0.029	0.033	0.036	0.028	0.040	0.014	0.031	0.028	0.033	0.023	0.028	0.026	0.023
PBU	0.049	0.052	0.034	0.039	0.041	0.046	0.038	0.050	0.021	0.036	0.035	0.044	0.033	0.038	0.026	0.028
PCF	0.045	0.048	0.028	0.031	0.031	0.039	0.031	0.042	0.017	0.031	0.032	0.039	0.028	0.033	0.022	0.023
PMC	0.055	0.057	0.040	0.026	0.032	0.031	0.031	0.035	0.014	0.036	0.018	0.023	0.014	0.019	0.039	0.031
PSA	0.054	0.057	0.038	0.031	0.036	0.034	0.033	0.039	0.017	0.037	0.022	0.030	0.020	0.024	0.035	0.029
PSV	0.043	0.045	0.031	0.020	0.020	0.027	0.018	0.029	0.013	0.032	0.026	0.027	0.018	0.023	0.023	0.024
PVE	0.064	0.059	0.046	0.017	0.017	0.016	0.019	0.016	0.015	0.047	0.028	0.020	0.015	0.013	0.044	0.039

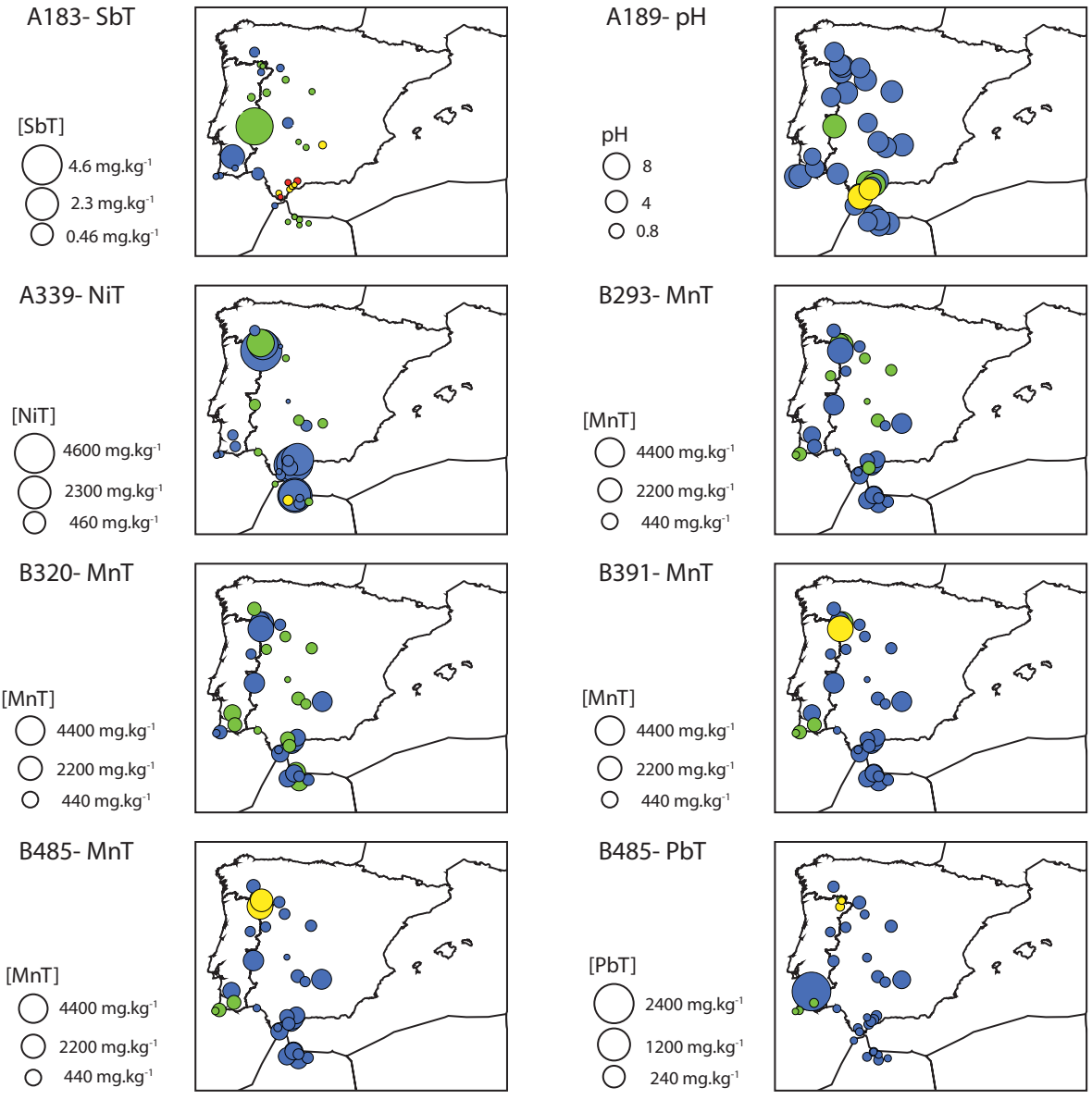
Supplementary material S 6.2: (continued)

	ETO	EVC	MBH	MBT	MEJ	MKE	MSI	MSII	MTA	PAL	PBR	PBU	PCF	PMC	PSA	PSV	PVE
EAC	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
EAL	0.9	0.487	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.735	0.9	0.309	0.655	0.9	0.9	0.744	0.9
EBE	0.854	0.591	0.464	0.518	0.9	0.499	0.569	0.637	0.427	0.57	0.606	0.643	0.608	0.669	0.669	0.621	0.669
ECA	0.805	0.296	0.9	0.683	0.9	0.9	0.9	0.9	0.9	0.22	0.453	0.433	0.499	0.669	0.669	0.552	0.669
ECO	0.9	0.302	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.537	0.605	0.187	0.187	0.487	0.487	0.293	0.487
ECR	0.834	0.547	0.9	0.35	0.9	0.9	0.9	0.9	0.9	0.349	0.14	0.685	0.414	0.293	0.293	0.35	0.293
EDE	0.854	0.23	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.343	0.452	0.297	0.252	0.487	0.487	0.324	0.487
EFS	0.854	0.23	0.9	0.517	0.9	0.9	0.9	0.9	0.9	0.343	0.452	0.297	0.252	0.487	0.487	0.324	0.487
EGJ	0.9	0.354	0.9	0.409	0.9	0.9	0.9	0.9	0.9	0.521	0.534	0.315	0.056	0.364	0.364	0.164	0.364
EGR	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
EGU	0.9	0.356	0.9	0.753	0.9	0.9	0.9	0.9	0.9	0.637	0.782	0.108	0.471	0.744	0.744	0.569	0.744
EMA	0.9	0.596	0.9	0.683	0.9	0.9	0.9	0.9	0.9	0.693	0.728	0.566	0.566	0.669	0.669	0.593	0.669
EMF	0.9	0.487	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.735	0.9	0.309	0.655	0.9	0.9	0.744	0.9
ERB	0.9	0.323	0.9	0.464	0.9	0.9	0.9	0.9	0.9	0.526	0.569	0.25	0.123	0.427	0.427	0.23	0.427
ESB	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
ESP	0.364	0.814	0.744	0.685	0.9	0.754	0.775	0.798	0.735	0.809	0.775	0.827	0.756	0.735	0.735	0.744	0.735
ETO		0.854	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.787	0.805	0.9	0.9	0.9	0.9	0.9	0.9
EVC	0.032		0.9	0.624	0.9	0.9	0.9	0.9	0.9	0.415	0.552	0.31	0.386	0.605	0.605	0.456	0.605
MBH	0.030	0.043		0.753	0.744	0.204	0.23	0.386	0.204	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MBT	0.027	0.016	0.042		0.9	0.762	0.782	0.803	0.744	0.605	0.464	0.669	0.364	0.204	0.204	0.285	0.204
MEJ	0.031	0.053	0.008	0.042		0.744	0.546	0.536	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MKE	0.023	0.038	0.003	0.037	0.006		0.295	0.321	0.293	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MSI	0.037	0.055	0.013	0.050	0.008	0.005		0.273	0.427	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MSII	0.031	0.049	0.006	0.048	0.005	0.003	0.001		0.536	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
MTA	0.045	0.063	0.028	0.059	0.027	0.031	0.036	0.029		0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
PAL	0.016	0.015	0.024	0.021	0.030	0.020	0.037	0.033	0.042		0.257	0.593	0.52	0.585	0.585	0.53	0.585
PBR	0.026	0.021	0.029	0.026	0.038	0.026	0.044	0.041	0.042	0.001		0.717	0.507	0.427	0.427	0.464	0.427
PBU	0.032	0.027	0.031	0.037	0.042	0.031	0.048	0.045	0.043	0.004	0.000		0.37	0.655	0.655	0.471	0.655
PCF	0.028	0.021	0.026	0.031	0.041	0.026	0.045	0.043	0.043	0.001	0.000	0.000		0.309	0.309	0.108	0.309
PMC	0.034	0.012	0.048	0.028	0.054	0.042	0.057	0.052	0.063	0.008	0.007	0.013	0.013		0	0.204	0
PSA	0.034	0.015	0.039	0.031	0.049	0.035	0.054	0.049	0.056	0.002	0.000	0.005	0.004	0.000		0.204	0
PSV	0.026	0.017	0.034	0.022	0.041	0.031	0.047	0.046	0.046	0.006	0.000	0.004	0.000	0.008	0.004		0.204
PVE	0.044	0.019	0.056	0.022	0.062	0.053	0.070	0.063	0.066	0.031	0.032	0.043	0.033	0.035	0.036	0.021	

Supplementary material S 6.3: Results of GEE analyses in *Cistus ladanifer*. All loci which correlated at least with one soil variable in the sampling area are given. QIC is the quasi-likelihood under the independence model criterion for GEE. The best models were of the kind of $\beta_0 + \beta_1 \cdot Variable_1 + \beta_2 \cdot Variable_2$.

Locus	QIC	β_0	$Variable_1$	β_1	$P\text{-value}_1$	$Variable_2$	β_2	$P\text{-value}_2$
A153	78.1	$3.95 \cdot 10^{15}$	**PbT**	$-8.71 \cdot 10^{11}$	0			
A183	319.6	-2.4	**SbT**	-0.23	$4.20 \cdot 10^{-7}$			
A189	112.8	-12.61	**pH**	1.52	$8.66 \cdot 10^{-7}$	**ZnT**	0.03	$1.29 \cdot 10^{-5}$
A245	286.1	-1.78	**SbT**	0.23	$4.03 \cdot 10^{-12}$			
A339	198.5	1.6	**NiT**	-0.002	$7.09 \cdot 10^{-6}$			
A492	123.3	-2.66	**NiT**	-0.002	$1.31 \cdot 10^{-6}$			
B152	121.5	4.6	**NiT**	-0.002	0.003			
B169	202.2	-1.0	**MnT**	0.002	$6.25 \cdot 10^{-8}$	**ZnT**	-0.04	$8.03 \cdot 10^{-5}$
B217	66.2	3.60	**SbT**	-0.60	$3.74 \cdot 10^{-11}$			
B293	122.9	-2.90	**MnT**	-0.002	0.001			
B320	187.1	-2.07	**MnT**	-0.002	0.001	**ZnT**	0.02	$5.69 \cdot 10^{-4}$
B346	238.4	-1.44	**SbT**	0.30	$3.24 \cdot 10^{-9}$			
B391	43.0	-3.1	**MnT**	0.001	0.001	**SbT**	-47.13	$8.95 \cdot 10^{-9}$
B401	178.2	-2.28	**MnT**	0.001	$4.43 \cdot 10^{-7}$			
B450	76.0	3.7	**SbT**	-63.64	$2.86 \cdot 10^{-5}$			
B485	70.0	-1.7	**MnT**	0.001	0.002	**PbT**	-0.09	$1.74 \cdot 10^{-4}$

Supplementary material S 6.4: Geographic distribution of loci potentially related with soil variables. For each locus we present a map where the geographic distribution of the band presence overlays the spatial variation of the soil variable with which it is correlated. The diameter of each circle is proportional to the value of the concerned soil variable in each population. The colour of the circle represents for each population the frequency (***f***) of plants in which the AFLP-fragment is present: blue (***f*** = 0); green (0 < ***f*** ≤ 0.50); yellow (0.50 < ***f*** ≤ 0.75) and red (0.75 < ***f***). Only bands which resulted in a Mann-Whitney test with a ***P*** lower than 0.05 are shown in the maps.



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